ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL CANCER INSTITUTE

FISCAL YEAR 1972
PART III

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE NATIONAL INSTITUTES OF HEALTH

ANNUAL REPORT

OF

PROGRAM ACTIVITIES

U.S. NATIONAL CANCER INSTITUTE

Fiscal Year 1972

Part III

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NATIONAL CANCER INSTITUTE

ANNUAL REPORT

EXTRAMURAL ACTIVITIES

July 1, 1971 through June 30, 1972

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ASSOCIATE DIRECTOR, EXTRAMURAL ACTIVITIES
NATIONAL CANCER INSTITUTE

The Extramural Activities area, an integral part of the total program of the National Cancer Institute, is responsible for the planning, developing, evaluation, and administration of national and international programs for support of cancer research and training through Federal grants and awards.

National Cancer Act of 1971

On December 23, 1971, the President signed the National Cancer Act of 1971, which authorizes the Director of the NCI, with the advice of the National Cancer Advisory Board, to plan and develop an expanded, intensified, and coordinated cancer research program encompassing the programs of the NCI, related programs of the other research institutes, and other Federal and nonfederal programs. Section 407 of the Act bears most directly on the Extramural Activities area.

Also inculded under the Act is authority for support of appropriate manpower programs of training in fundamental sciences and clinical disciplines to provide an expanded and continuing manpower base from which to select investigators, physicians, and allied health professions personnel, for participation in clinical and basic research and treatment programs relating to cancer, including where appropriate, the use of training stipends, fellowships, and career awards.

The Act also provides for the establishment of 15 new centers for clinical research, training, and demonstration of advanced diagnostic and treatment methods relating to cancer.

Grants Not Exceeding $35,000

The Act provides that under procedures approved by the Director, National Institutes of Health, the Director, National Cancer Institute, may approve grants for cancer research or training in amounts not to exceed $35,000 after appropriate review for scientific merit, but without the review and recommendation of the NCAB.

All competing applications requesting funds in the amount of $35,000 direct costs or less for the initial budget period are automatically eligible for an early award. Eligible applications that receive a favorable recommendation with a suitable priority from an appropriate initial review group may be issued an early award upon selection by the responsible program director and after approval of the Director, NCI. Eligible applications which have been favorably recommended by the initial review group but which have a priority insufficient to permit an award will not be eligible for an award and the applicant will be notified in accordance with usual procedures.

An early award will represent the level of support required for an initial budget period not to exceed $35,000 direct costs and for subsequent consecutive budget periods which do not exceed this level. In the case of those

applications in which future budget periods recommend levels of support in excess of $35,000 the notification of early award will not show commitments for those budget periods in excess of this level. A list of these early award grants will be submitted to the NCAB at its next meeting for their recommendation for future commitments including general level of support and total project period.

Cancer Control Program

The Act authorizes the establishment of programs as necessary for cooperation with State and other health agencies in the diagnosis, prevention, and treatment of cancer, with $20 million authorized to be appropriated for the fiscal year ending June 30, 1972. At the first meeting of the National Cancer Advisory Board on March 20-22, the Chairman appointed a Subcommittee on Cancer Control. It will be the purpose of this subcommittee to define the area, evaluate objectives and set criteria for carrying out the program, and advise the Director, NCI. Meetings have been held with various groups and agencies within the Government so that there will be no overlap in the Cancer Control Program as developed by the NCI.

Construction

In keeping with the expanded, intensified, and coordinated plan for the conquest of cancer, an initial amount of $11 million was included in the $100 million additional monies requested by the President, and approved by the Congress, to provide additional facilities and to develop new, strong, multidisciplinary cancer efforts in regions of the country where they do not now exist. Matching funds from local sources will be required with the Federal Government providing up to 75 percent.

Planning for Cancer Centers

A two-day conference sponsored by the American Cancer Society and the NCI in cooperation with the Association of American Medical Colleges was held in December 1971 at the Washington Hilton Hotel and drew some 700 professionals from the scientific community throughout the country. The first morning session was devoted to the Objectives and Problems of Developing and Operating Cancer Centers, and reports on Education and Training, Research, Service, and the Administrative and Fiscal View, presented by speakers from the scientific community. The second morning session, chaired by Dr. J. Palmer Saunders, the Associate Director, EA, NCI, presented the NCI's Role in the Development of Cancer Centers, with staff members reporting on (1) Current National Cancer Institute Center Programs, (2) The Role of the Cancer Center in Training, (3) Planning for Cancer Centers, (4) Construction and Renovation, and (5) Review of Proposals. The afternoon sessions, both days, were given over to workshops on the topics discussed in the morning sessions, with extramural staff of the NCI acting as moderators on the first day while Center grant awardees acted as consultants; on the second day, institution scientists were the moderators with the staff of the NCI as consultants.

Evaluation of Cancer Research Outside the Continental United States

During the past year, the Deputy Associate Director for EA, NCI, Dr. William A. Walter, Jr., accompanied by Dr. R. Lee Clark, Jr. of the M. D. Anderson Hospital and Tumor Institute, Houston, visited Copenhagen, Denmark; Helsinki, Finland; Stockholm, Sweden; Oslo, Norway, and Frankfort, West Germany, to obtain some insight into experience with centralized cancer facilities. They were also interested in the utilization of centralized cancer care facilities for the training of research personnel and sought information about the interface between cancer research activities and clinical care of cancer patients. The visitors concluded that the various countries utilized available resources in different fashions, depending in large part upon the specific economic and cultural traditions which prevailed.

In November 1971, Dr. John T. Kalberer, Jr., Special Assistant to the Associate Director, EA, NCI, and Dr. Patrick J. Cavanaugh, Professor of Radiology and Chief of Radiation Therapy, Duke University Medical Center, Durham, North Carolina, visited five Eastern European countries principally to examine cancer research, diagnosis, and therapy as it is conducted within the framework of the national health systems in the countries concerned. They met with the Ministers of Health in the principal cities of Budapest, Belgrade, Bucharest, Prague, and Warsaw, and also visited medical institutions in the cities of Cluj, Romania; Ljubljana and Zagreb in Yugoslavia; Debrecen, Hungary; and Bratislava in Czechoslovakia. It was apparent to them that the East Europeans are anxious to have their scientists spend time in the United States for training in all aspects of cancer research but particularly in the use of sophisticated pieces of equipment which involve professional and technically trained people, such as physicists, dosimetrists, and supportive technicians.

National Organ Site Program

The principle of grant supported programs in which planned and integrated efforts are coordinated at a headquarters institution other than the National Cancer Institute got underway during this past fiscal year. Approved by the National Advisory Cancer Council at its June 1971 meeting, this principle involves a departure from the usual practice of traditional operations of support of biomedical research while retaining the dual review aspects of grant supported activities. The program will support applications for several projects conforming to the approved National plan.

Subcommittees have been appointed and planning activities have been initiated for projects in Bladder Cancer, Large Bowel Cancer, and Prostatic Cancer. Dr. Samuel Price has been appointed Project Administrator to administer the special targeted area programs.

Biohazards Control

In the past two years, the NACC, as well as several other NIH advisory groups, have been concerned with the increased use in experiments of biological or chemical materials that may result in the exposure of laboratory personnel and the general public to potential hazardous agents. After passing a

resolution at its June 1971 meeting, the NACC requested that these specific problems be brought to the attention of the Director, NIH. It was felt that more systematic attention should be accorded to the problem. Consequently, the matter was considered by the Subcommittee on Research of the NIH Executive Committee on Extramural Affairs. Its recommendations were considered and unanimously accepted by the ECEA. The following recommendations are to be brought to the attention of the NIH Board of Advisors:

1. That the Office of the Director, NIH, issue a memorandum to all Health Scientist Administrators in the extramural programs of the Institutes and Divisions calling their attention to the problem; that before an award is made on an approved application where a biohazard has been identified, an assurance should be received from the investigator and his institution indicating that they are aware of the hazard and that appropriate safety precautions, as determined by the institution, are being utilized.

2. That the NIH, in collaboration with the National Academy of Sciences, as soon as possible, study in more detail the potential hazards of research involving infectious agents and chemical carcinogens, mutagens, and other highly toxic substances, and organize a conference or a series of conferences, and publications, to make the scientific community aware of the problems involved and safety procedures needed.

3. That an item be published in the Division of Research Grants Newsletter informing our grantee community that NIH is concerned and currently studying the problems associated with the use of these materials in research projects; further, that instructions accompanying the application form PHS-398 should include a section on biohazards and request the investigator to describe the safety procedures adopted by the applicant's laboratory when a hazardous agent is being used in the research project.

A brochure, "Biohazard Control and Containment in Oncogenic Virus Research" together with a letter explaining the concern of the NCI in this matter, is routinely sent to appropriate new grantees as awards are made.

Cancer Core Enrichment Program

A program is being developed, designed to assist qualified institutions and organizations within the territorial United States to plan and develop activities which will lead to earlier diagnosis and more successful management of the common forms of cancer. Its purpose is to (1) determine defects within existing patterns or systems of health care with regard to early detection and/or diagnosis of cancer; (2) determine what remedial steps may be initiated to correct and repair these defects; and (3) effect, within a given area, a demonstrable reduction in cancer morbidity and mortality.

Budget

Grant activities in fiscal year 1972 increased markedly over fiscal year 1971. Obligations for all grants in fiscal year 1971 totalled $110 million while the estimate for fiscal year 1972 is $152 million, an increase of 38 percent. In

fiscal year 1971, 2,236 applications requesting $135 million were reviewed. In fiscal year 1972, 2,868 applications requesting $264 million were reviewed, an increase of 28 percent in number of applications and 96 percent in dollars, respectively. Even with a substantial increase in appropriations, however, the number and amount remaining unfunded increased. In fiscal year 1971, 434 approved applications, totalling $17 million, were not funded, while in fiscal year 1972, the estimate is approximately 580 at $55 million, an increase of 34 percent in number of grants, and 224 percent in dollar amount, respectively.

Training

Funds available for both graduate and clinical training programs have been less than required for program needs. The total training dollars for fiscal year 1971 amounted to $10.8 million. The President's budget for fiscal year 1972 allotted a total amount of approximately $8.4 million; an additional amount of $1.8 million for training was included in the $100 million additional monies requested by the President, and approved by the Congress. After the fiscal year started, the Director, NCI, allotted an additional $300 thousand to the training area in December 1971. In order to start additional new training programs in fiscal year 1972, graduate training awards were negotiated at approximately 30 percent below recommended levels and clinical training grants at about 7 percent below recommended levels.

Seventy percent of the monies awarded in the graduate research training area have supported stipendiary funding, the balance being available for operational costs or professional salaries. While the other institutes at NIH did not receive additional money for training, the additional funds allotted to the NCI training area were used in fiscal year 1972 for clinical training to fund 14 approved applications from fiscal year 1971.

Additional data and comparisions are summarized in the two tables given below:

Research Grants (000's)

	FY 1970	FY 1971	FY 1972	FY 1973
Requested Direct Costs	$101,290	$111,999	$174,007	$245,023
% Increase (Decrease)		+11%	+55%	+41%
Approved Direct Costs	83,205	88,463	120,433	172,026
		+ 6%	+36%	+43%
Requirements	101,925	106,248	149,688	219,084
		+ 4%	+41%	+46%
Awarded	78,462	95,317	125,311	160,466
		+22%	+32%	+28%
Unfunded	23,463	10,931	24,377	58,618
		-53%	+123%	+41%

Research Grants (000's) (continued)

	FY 1970	FY 1971	FY 1972	FY 1973
NCI Budget	$191,091	$232,853	$337,599	$430,000
% Increase		+22%	+45%	+27%
Research Grants	$ 78,462	$ 95,317	$125,311	$160,466
% of NCI Budget	+41%	+41%	+37%	+37%

FY 1971 Versus FY 1972
Increases in Major Research Grants Programs (000's)

New Applications

Requested	% Increase Numbers	Change	% Increase Dollars	Change
Traditional	88%	+444	116%	+ $22,803
Program Centers	109%	+ 12	173%	+ 9,140
Exploratory Projects	136%	+ 19	214%	+ 2,488
Clinical Centers	100%	+ 4	419%	+ 8,391
Total		+479		+ $42,822
Approved				
Traditional	80%	+263	91%	+ $ 8,820
Program Centers	67%	+ 6	129%	+ 3,602
Exploratory Projects	167%	+ 20	300%	+ 2,443
Clinical Centers	250%	+ 5	588%	+ 4,240
Total		+294		+ $19,105
Awarded				
Traditional	110%	+184	166%	+ $ 9,367
Program Centers	13%	+ 1	67%	+ 2,117
Exploratory Projects	-25%	- 3	3%	+ 24
Clinical Centers	200%	+ 5	294%	+ 2,638
Total		+187		$14,146

Equal Employment Opportunity

The Grants Administration Branch has continued its efforts to improve the opportunities for the growth and advancement of its minority group employees. The main thrust of this effort is directed toward improved communication between the employees and supervisors. Minority employees at the GS-7 level

and below have been encouraged to hold discussion groups in order to identify grievances, problems, and concerns, and to suggest improvements. Meetings between supervisors and the group leaders from these discussion groups have resulted in the following actions or plans: (1) Job descriptions of all positions at the GS-7 level and below are being reviewed and updated where needed; (2) minority employees are encouraged to seek individual counseling from their supervisors and personnel representative in career growth and development; (3) a seminar for minority employees at which representatives from personnel and the training office will discuss those specific areas where additional information is needed; and (4) minority employees have been encouraged to participate in the Upward Mobility Program by enrolling in training programs and by taking advantage of courses in local colleges. To date, three Grants Administration Branch employees are participating in the Upward Mobility Program; in addition, over the past two years, there have been 58 enrollments in various training courses. Guidelines have been developed to assist supervisors in selecting employees for course enrollment when scheduling problems arise.

Staff Changes

Continuing the precedent established in the Radiation Therapy Program, a guest scientist on sabbatical leave was again given the direction of the program for fiscal year 1972. Dr. Raul Mercado, Associate Professor of Clinical Radiology and Associate Director of Radiation Therapy at the University of Pittsburgh School of Medicine and Medical Center, respectively, is currently serving in this capacity. Because of the recent increase in developmental and planning efforts in the radiation area, Radiation Biology and Physics are now under the direction of Dr.Francis J. Mahoney.

In the Centers Program area, Dr. Genrose Copley has been appointed co-director with Dr. William L. Roberson. Mrs. Patricia Von Bargen has recently joined the Centers Program as a program assistant and shortly, Mrs. Cynthia Corangi will join the staff in the same capacity.

Dr. Kenneth C. Potter is the new Program Director for the Graduate Research Training Program, and is also the Executive Secretary of the Cancer Research Training Committee. He replaced Dr. Arthur L. Schipper, who has taken on a new assignment as the Executive Secretary of the Cancer Research Center Review Committee. Dr. Schipper replaced Dr. George E. Jay, who has become the Program Director of the newly created NCI Cancer Research and Training Facilities Construction Program. Dr. Jay is in the process of hiring staff to implement the activities of this program. Mr. Manfred C. Massa has already joined his staff and it is expected that additional personnel with architectural and engineering backgrounds will join the program shortly.

Mr. Leroy Houser, Program Specialist, has joined the Awards Review and Technical Administration Branch. In the Grants Administration Branch, Mr. Patrick Talmon has been named Head, Grants Management Section.

In addition to the above personnel changes, there are several pending accessions, including that of Dr. John Lane as Special Assistant to the Associate Director, and Dr. Robert Manning, also in the Office of the Associate Director.

PROGRAM ANALYSIS AND REPORTING SECTION

The Program Analysis and Reporting Section (PARS) is located administratively in the Office of the Associate Director for Extramural Activities, NCI, and is dedicated to the collection, storage, analysis and reporting of scientific information generated primarily by the extramural programs. Information is also developed which is concerned with NCI contract supported research and intramural investigations. The major thrust of this group is research analysis, directed at the scientific content of the different programs. While certain administrative data, e.g., amounts awarded, are maintained and compiled, in order to demonstrate program financial emphasis, science remains the prime concern and interest of this office.

Data related to the various programs are compiled from applications, proposals, program review statements, progress reports and scientific papers published by extramurally-supported scientists. During the past twelve months the Section received over 350 requests for special information. These requests range from special data required to support Congressional Budget Hearing statements, to information related to NCI support in a specific research area.

Certain information is readily available and quickly compiled and is used to answer recurring type requests, e.g., NCI support related to smoking. However, other requests are of a one-time nature and may require many man hours of effort. Typical of such a request is one entitled, "From Fundamental Science to Applied Clinical Cancer Diagnosis and Treatment." This was requested by the Director, NCI, to emphasize the importance of continued support for fundamental basic science. Nine separate reports were finally developed and individually entitled:

Combination Drug Therapy for Acute Leukemia
The Cobalt Isotopes
Alkylating Agents
Early Detection of Cervical Cancer by the Cytologic Technique of Papanicolaou
Carcinoembryonic Antigen of the Human Digestive System
Purine and Pyrimidine Antimetabolites
Methotrexate and Choriocarcinoma
Supervoltage Radiotherapy
Serum Acid Phosphatase and Cancer of the Prostate

Another similar study was made requiring documentation of specific NCI projects (intramural, grants, and contracts, both clinical and basic) which have derived direct benefit from fundamental research supported by grants from NIGMS.

The Section is continuing the listing of grantee published papers in the form known as the "PARS Digest." This publication contains the reference for each paper published during the previous month in over 350 select journals, which acknowledges NCI extramural support. The references are arranged within program areas (carcinogenesis, epidemiology, etc.). Each reference is also indexed in more depth by a special indexing system, which permits greater selectivity. Approximately 3400 references are processed each year and included in the PARS Digest.

In the past, the Section used to abstract many of the papers, which were published by grantees; this collection of abstracts was known as "PARS Digest, Part I." Following a survey of extramural program directors last year, it was decided to discontinue the abstracting operation in favor of more detailed in-depth reports on specific subjects. The subjects of the reports are selected after consultation with program directors and others, or as the state-of-the-art would indicate. Four reports have been completed to date. They are entitled:

In Vitro Reversion of Neoplastic Transformation
Normal and Tumor Immunology and Current Immunotherapy
Prostaglandins and Cancer
Cyclic 3', 5' Adenosine Monophosphate and Cancer

Two other reports are in the final stages of preparation:

Biochemical Markers in Cancer
In Vitro Screening for Chemical Carcinogens

The Section maintains data on NCI supported projects from their inception through their completion. This provides a unique opportunity to survey NCI's involvement in a given area of cancer research as evidenced by the published papers of its extramural awardees, progress reports of ongoing projects, and research proposals of new applications for research support. All of these data will be utilized to the fullest extent in preparing narrative reports on current developments in cancer-related research. Since these reports will include unpublished material, when available, their circulation will be limited.

The National Cancer Act of 1971 states that the Director shall establish an international cancer research data bank to collect, catalog, store, and disseminate results of cancer research. Staff of PARS have participated, along with other NCI groups, in the initial planning stages for such a data bank. It is anticipated that this operation could conceivably require many man hours of thought and labor before it is functionally operational.

In an attempt to increase the dissemination of research information within the NCI, PARS has expanded one operation and instituted a new one. The distribution of grantee reprints has been enlarged to include all three major research components of NCI, Chemotherapy, Etiology, and General Laboratories and Clinics. The Research Information Branch, OD, also receives reprints. PARS should receive five reprints of each paper published by NCI grantees, one is maintained as an "Official Copy." The new operation involves distribution of abstracts of the awarded research grants, which are written by the grantee and submitted with his grant application. These abstracts are prepared for all new grant applications and all competing renewals. The abstracts, like the reprints, are being sent to Chemotherapy, Etiology, and General Laboratories and Clinics. It is hoped that both receive wide distribution among the NCI staff.

The NCI and the NIDR have joined forces to develop an ongoing survey of persons who have received training under either a Graduate Training Grant or a Fellowship. The survey is to provide data that would assist in the evaluation of

the training programs. The major portion of this work is proposed to be performed by an outside contractor. Thirty organizations responded to the initial request for resumes of experiences and capabilities. Staff of PARS participated in the selection of eight of them to receive a request for a proposal (RFP). The proposals have been submitted and an ad hoc committee is presently reviewing them. A great deal of data on the trainees have been compiled by PARS which will be turned over to the contractor once the selection is made. It is anticipated that the contractor will continue to require NCI assistance in performing its duties and that PARS will supply much of this support.

The Section has continued serving as a central screening point to detect conflict between proposed grant and contract support. The purpose of this operation remains to alert the responsible program areas to a possible duplication of either workscope or budget between grants and contracts.

Each extramural project is reviewed by PARS prior to award to determine if human experimentation is involved in the research proposal. Each grantee institution wherein human experimentation is conducted and supported by grant funds is required to present evidence of institutional review and approval of such research. Over the years there has been a gradual evolution of thought regarding what is and what is not considered human experimentation. This continual alteration of moral judgment standards has complicated the review process.

NIH has developed new requirements, similar to the human experimentation standards, for all grantees and contractors who utilize any one of six types of warm-blooded animals in research projects (dogs, cats, monkeys, guinea pigs, hamsters, and rabbits). These researchers are required to assure the NIH that they will evaluate their animal facilities in regard to the maintenance of acceptable standards for the care, use and treatment of such animals. This section will have the responsibility of identifying extramural supported projects where any of these types of animals are employed.

At the request of the Associate Director for Program Planning and Analysis, NCI, staff of PARS reviewed the final draft of the National Cancer Plan. This review was for scientific content and was conducted in the offices of Auerbach Associates, Inc. As the review proceeded it became apparent that changes should be made in the scientific narrative of the report. Written comments were submitted to the committee, which prepared the report, for its review and use in revising certain sections.

Appendix I, which appears at the end of this report has been prepared by the Program Analysis and Reporting Section. This comprehensive narrative review attempts to highlight certain areas of research, the results of which have been published during the past year by investigators who received NCI extramural support.

Statistical Summary of NCI Extramural Awards

	F. Y. 1971 (Actual)		F. Y. 1972 (Estimate)	
	Number Projects	Amount	Number Projects	Amount
A. Research Grants				
Total Research Grants	1,096	$ 89,416,000	1,400	$ 130,259,000[1]
General Research Support	- -	5,901,000	- -	6,052,000
B. Training				
Postdoctoral Fellowships	145	1,163,000	110	838,000
Special Fellowships	46	623,000	56	759,000
Graduate Training Grants	86	5,317,000	95	6,217,000
Clinical Cancer Training Grants	105[2]	5,457,000	110	6,657,000
C. Research Career Program				
Research Career Awards	11	331,000	11	331,000
Research Career Development Awards	71	1,681,000	74	1,620,000
Grand Total	1,560	$ 109,889,000	1,856	$ 152,733,000

[1] Includes Construction Grants
[2] Includes Grants Active by Extension

CARCINOGENESIS PROGRAM

The Carcinogenesis Program is concerned with the achievement of a more effective prevention of cancer in man, caused or promoted by chemical or physical agents, or by the possible interaction of chemical carcinogens with biological agents. Representative areas of principal interest to the program are the development of biochemical approaches to the identification of pre-cancerous and early cancerous lesions, the construction of *in vitro* systems for the identification of chemical cancer-producing substances, and the development of methods (e.g., chemical, hormonal, dietary) for modifying host response to carcinogens. The program also supports a large amount of research on the biochemistry of host-tumor relationships, as well as basic biochemical studies of potential significance to cancer research. By means of graduate training grants, the program seeks to attract talent into carcinogenesis research and to provide for the further development of carcinogenesis research personnel.

The carcinogenesis program supports a total of 321 grants, consisting of 309 research grants costing $21,948,818 and 12 graduate training grants at a cost of $1,145,176. The total cost of all 321 grants is $23,093,994.

The several categories of research included in the carcinogenesis program are further described in the outline shown below.

CARCINOGENESIS PROGRAM

(309 research grants, $21,948,818)

I. Chemical Carcinogenesis (86 grants, $11,183,753)

1. Isolation, identification, and synthesis of known and suspect chemical carcinogens and their metabolites; molecular structure - carcinogenicity relationships
2. Action, mechanism of action, and physiological disposition of chemical carcinogens: Biochemical changes in physiological compounds and processes produced by chemical carcinogens. Cytologic and histologic changes produced by chemical carcinogens; effects on cell ultrastructure and function. Properties of cells transformed by chemical carcinogens; development of carcinogenicity screening procedures. Carcinogenicity screening. Metabolism, toxicity, and excretion of chemical carcinogens and their metabolites; identification of proximate carcinogens. Factors which initiate, promote or inhibit the action of chemical carcinogens; influence of age, sex, species, and strain. Metabolic patterns in preneoplasia and neoplasia.

This category (I) includes 77 project grants (R01), eight Program Centers (P01), and one Clinical Center (P02). Fiscal year 1972 costs are $3,519,580 for the project grants and $7,664,173 for the combination of Program and Clinical Centers.

II. Biochemistry of Cancer and Cancerous Hosts (136 project grants, $6,329,576)

1. Carbohydrates, Energy Metabolism, and Respiration of Cancer Cells, and Cancerous Hosts
2. Amino Acids, Proteins, and Ribosomes in Cancer
3. Lipids in Cancer
4. Nucleic Acids in Cancer
5. Endocrine-Related Biochemistry of Cancer and Cancerous Hosts
6. Enzymes in Cancer
7. Metabolic Patterns in Preneoplasia and Early Neoplasia
8. Development of Biochemical and Biophysical Methods for the Early Detection of Cancer
9. Biochemistry of Melanin in Melanoma and in Other Systems

III. Basic Biochemistry of Potential Significance to Cancer Research (87 project grants, $4,435,489)

1. Biochemistry and Biophysics of Amino Acids and Proteins, Biosynthesis of Proteins
2. Biochemistry and Biophysics of Nucleic Acids and Related Compounds
3. Endocrinology with Potential Applications to Cancer
4. Enzymes: structure, synthesis, regulatory properties, mechanism of action.
5. Other Biochemistry of Potential Significance to Cancer Research

In this report, all references to costs are in terms of total costs, either actual or estimated. Of the project grants (R01) recommended for funding by the National Cancer Advisory Board at its March 1972 meeting, it is currently projected that at least 40 percent of the new (Type 1) applications and 70 percent of the competitive renewal (Type 2) applications will be funded from fiscal year 1972 funds and these are included in the totals shown in the present report. No analogous assumption is made with respect to possible fiscal year 1972 funding of any of the existing backlog of eligible, currently unfunded program center (P01) applications.

The past year has seen a rapid expansion in the number (+29 percent) and cost (+50 percent) of research grants in the Carcinogenesis Program. This includes: (a) an increase from 232 to 300 project grants, with an attendant increase in funding from $8.8 million to $14.3 million, and (b) an increase from seven to nine Centers, with a $1.9 million increase in funding. Although these

differences are not absolute as pertains to a comparison of fiscal year 1971 and fiscal year 1972 annual reports (due to differences in cut-off dates), they do properly portray the pattern of rapid growth in program, resultant from the expanded national emphasis on cancer research.

Categories I (Chemical Carcinogenesis) and II (Biochemistry of Cancer and Cancerous Hosts) represent categorical research, whereas Category III (Basic Biochemistry of Potential Significance to Cancer Research) constitutes the non-categorical component of the Carcinogenesis Program. In this context, approximately 71 percent of the project grants in this program now represent categorical research, an increase of about 10 percent during the past year.

Program Highlights

Dr. E. Boyland in 1950, and others since that time, have suggested that epoxides are intermediates in the metabolism of carcinogenic hydrocarbons. However, until the recent work of Dr. Charles Heidelberger's group (CA7175), this had not been demonstrated. By the use of heated microsomes to inhibit epoxide hydrase, short incubation times and relatively large amounts of unlabeled epoxide to trap labeled epoxide which might be formed as a result of the incubation of rat liver microsomes with labeled dibenz(a,h)anthracene (DBA), the Heidelberger group was able to identify one or more epoxides as being produced in the microsomal metabolism of DBA.

Dr. Heidelberger (CA7175), in conjunction with workers at the Chester Beatty Institute, have studied the *in vitro* transformation of hamster embryo cells by K-region derivatives of carcinogenic hydrocarbons, principally benz(a)-anthracene (BA) and dibenz(a,h)anthracene (DBA). They found that cell transformation by the parent hydrocarbons was not significantly different from that of the control (acetone), that the K-region epoxides were more effective, and that increasing the concentration of the epoxide increased the frequency of transformation. K-region phenol derivatives were highly toxic but did not produce a significant frequency of transformation. These findings support the view that metabolism of the hydrocarbons studied is necessary to produce cell transformation and that transformation and toxicity are separate events.

Dr. R.G. Gould in 1951 showed that the conversion of acetate to cholesterol is strongly inhibited in liver slices from animals which had been fed a high cholesterol diet. Subsequently it was shown by Dr. Marvin Siperstein (CA08501) that this feedback mechanism is absent in a series of 14 rat minimal deviation hepatomas, white cells from a guinea pig leukemia, and aflatoxin-induced hepatomas of rainbow trout. Moreover, the trout data would appear to indicate that the loss of cholesterol feedback occurs at least nine to 12 months before the earliest pathological signs of malignancy. In preliminary studies the Siperstein group has shown that the cholesterol feedback mechanism is lost in the intact hepatoma-bearing rat, as well as in the *in vitro* system.

Siperstein et al. have also shown that the feeding of cholesterol for three days to rats produces a consistent, 90 percent reduction in the activity of HMG-CoA (hydroxy methyl glutaryl coenzyme A) reductase in normal liver,

whereas in hepatoma this enzyme did not show a significant decrease in activity after cholesterol feeding. The latter data provide direct evidence that HMG-CoA reductase is the enzymatic site of the cholesterol feedback lesion in cancer. This is the enzymatic step at which 3-hydroxy-3 methyl-glutarate is converted to mevalonate.

Loss of the cholesterol feedback system in hepatomas is impressively consistent and appears to be the sole example of generalized loss of a feedback system in malignant tissue. The question of whether this biochemical lesion is an integral part of the carcinogenesis process is of commanding interest.

Dr. Lee W. Wattenburg (CA09599) has been concerned with studies on the in vivo induction of benzpyrene (B.P.) hydroxylase activity and with determinations of the extent to which the induction of this microsomal enzyme system inhibits tumorigenesis in animals exposed to carcinogenic hydrocarbons. His data include the finding that the inclusion of B-Naphthoflavone in the diet of mice causes a five-fold increase in B.P. hydroxylase activity in the lung and that the subsequent oral (intubation) administration of benzo(a)pyrene (B.P.) resulted in an almost total inhibition of pulmonary adenoma formation. The feeding of quercetin pentamethylether, a less potent inducer of B.P. hydroxylase activity, lowered the inhibition of pulmonary adenoma to approximately 50 percent. Analogously, topical application of B-Naphthoflavone to the skin of mice resulted in a 23-fold increase in skin B.P. hydroxylase activity and a 50 percent reduction in skin tumor formation resulting from initiation by B.P. and promotion by croton oil. In these same studies it was observed that the feeding of B-Naphthoflavone to mice caused a 53-fold increase in B.P. hydroxylase in the small intestine.

Wattenberg (CA09599) together with Dr. George T. Bryan (CA08254 and CA10017), and others, have studied the protective action of phenothiazine in rats when added to a diet rendered carcinogenic by the inclusion of bracken fern. Phenothiazine is known to induce B.P. hydroxylase activity in the liver and small intestine mucosa of the rat. Nineteen of 21 rats (90 percent) fed a bracken fern containing diet (one part bracken fern and three parts grain) developed intestinal neoplasms (adenomatous polyps or adenocarcinoma) and the same number of animals developed urinary bladder tumors (papillomas or transitional cell carcinomas). When phenothiazine was added to the bracken fern-grain diet (2. mg. phenothiazine per gram of diet), 11 of 28 rats (39 percent) developed intestinal tumors and only 10 of the animals developed bladder tumors. In this study, then, phenothiazine caused a significant reduction in the incidence of intestinal and urinary neoplasms.

Wattenberg (CA09599) has shown that most and possibly all of the B.P. hydroxylase activity of the small intestine of the rat is due to inducer present in the diet. Animals starved for one day or longer showed no B.P. hydroxylase activity in the small intestine. Virtually the same was true in the case of animals fed a "purified" diet consisting of starch, vitamin-free casein, corn oil, salt mixture, and a complete vitamin supplement. In some cases the addition of an air-dried ground vegetable to the "purified" diet caused induction of small intestine B.P. hydroxylase activity.

Induction of B.P. hydroxylase activity, expressed in units per milligram of wet weight, was 23.8 for brussel sprouts, 11.6 for cabbage, and lesser amounts for turnip greens, broccoli, cauliflower, alfalfa, and spinach. The lettuce and artichokes tested did not show any inducing activity. These data appear to suggest that the amount of hydroxylase inducers in the diet might affect the organism's capacity to detoxify polycyclic hydrocarbon carcinogens.

CANCER BIOLOGY PROGRAM

The Cancer Biology Program is designed to provide fundamental information on the causes and nature of cancer in man, with the expectation that this will result in better methods of diagnosis, prevention, and treatment of neoplastic diseases. The program comprises the areas of Viral Oncology, Tumor Biology, and Cancer-related Biology.

Viral Oncology is concerned with the role of viruses in the etiology of cancer and the consequences of this for the identification of susceptible individuals, early diagnosis, and development of new modes of prevention and treatment. Investigations include studies at the cellular and molecular levels as well as the animal level. Research on the mechanism of carcinogenesis, gene expression, and other cell regulatory functions, utilizing viruses as tools, are an important part of this segment of the program.

In Tumor Biology the emphasis is on the preneoplastic and neoplastic lesion itself, on the tumor-host relationship in its various manifestations, and on the biology of the tumor cell.

Cancer-related Biology encompasses aspects of cell biology, molecular biology, embryology, genetics, morphogenesis, pathology, and other disciplines that may range beyond the confines of manifest cancer research, but contribute significantly to it, conceptually and experimentally. While the greatest interest resides in the biology of animal cell systems, projects which involve other forms of life, if these offer particular advantages, are also considered to be appropriate for the cancer biology program.

During fiscal year 1972, the Cancer Biology program consisted of 278 traditional grants ($16,100,325), nine Centers ($3,721,642), 14 graduate training grants ($1,016,102), one exploratory project ($15,176), 29 Postdoctoral Fellowships ($229,472), six Research Career Awards ($182,383) and 26 Research Career Development Awards ($602,802) for a total of 363 awards costing $21,867,902 (direct and indirect costs).

Of the 278 traditional grants, 105 were in the Viral Oncology area, of which 38 were concerned with the RNA viruses, two with the mammary cancer agent and 54 with the DNA viruses. In the Tumor Biology segment, 20 were involved with various aspects of the tumor-host relationship, 33 were in cell biology, 13 in

genetics, 26 in molecular biology and biochemistry, and the remainder in aspects of ultrastructure of cells, pathology,and related subjects. Eighty-two projects comprised the area of cancer-related projects distributed among various disciplines and research areas, such as microbiology, cell biology, genetics, pathology,and molecular biology.

Highlights

The reverse transcriptase of the RNA tumor viruses continues to provide some of the most fruitful developments in viral oncology and molecular genetics. Although the evidence is indirect and not unambiguous, the possibility still exists that this enzyme is necessary for malignant transformation. For example, there is a direct correlation between inhibition of this enzyme and of cellular transformation. Again, a certain non-transforming mutant of a transforming virus lacked the transcriptase, further strengthening the possibility of such a relationship.

Of more immediate interest, however, are the uses to which this enzyme has been put. A notable case concerns the identification of RNA tumor virus genetic material in human cancer cells. Using highly radioactive DNA transcribed by mouse mammary tumor (MTV) RNA, RNA specifically related to MTV RNA has been identified in human mammary cancer. Similarly, DNA transcribed by mammalian viral RNA served to detect a related RNA in human leukemia. The specificity of these results were stressed; that is, normal human tissue did not reveal such RNA, nor was there significant cross reaction between the RNA of human mammary cancer and of human leukemia.

A more recent use of these transcriptases is in the synthesis of specific cellular DNA sequences (genes?). Although reverse transcriptases have a high affinity for their natural template, that is, the associated tumor virus RNA, they also accept unrelated RNA. Using messenger RNA (mRNA) for human or rabbit globin synthesis, a complementary DNA was synthesized with properties suggestive of, or, at least closely related to the gene for globin synthesis. Clearly, this is the beginning of an important development in molecular genetics and will also provide further techniques for the analysis of malignant transformation.

In the cell, messenger RNAs (mRNAs) are transcribed in the nucleus and transported to the cytoplasm where they specify the synthesis of proteins, i.e., translate the message.

Recent evidence indicates that an adenylic acid moiety (PolyA) attached to mRNA is concerned with this transport. Indeed, the presence of sequences of Poly A in RNA is considered to be diagnostic of mRNA. It is, therefore, of considerable interest that Poly A is also found in a specific subunit of tumor virus RNA, indicating that this subunit may be a mRNA. This subunit may, therefore, contain the information for synthesizing the transforming gene (catalyzed by reverse transcriptase), or a transforming protein (catalyzed by cellular enzymes). It is significant that influenza virus, a budding membrane virus resembling the RNA tumor viruses (although non-oncogenic), does not contain such a moiety.

The DNA tumor viruses continue to command much attention, not only because they are or may be etiological agents of naturally-occurring neoplasia - as is certainly the case with certain of the herpes-viruses - but also because the smaller members, such as polyoma and SV40, are such simpler transforming agents relative to the RNA viruses, that it is hoped that information about the essential nature of malignant transformation and its expression will be gained more expeditiously. However, even with these simpler agents, far too many functions appear in cells they transform than can be ascribed to the viral genome. One approach that is being taken, therefore, is to develop cell-free systems to transcribe viral DNA (or its segments) to mRNAs, then translate these to specific proteins whose functions can be examined. The success with cell-free systems using reverse transcriptase, described above, encourages such an arduous undertaking.

Last year, viral gene functions were described that were responsible for the initiation and/or maintenance of transformation. Evidence has now been obtained that, at least in the case of SV40, maintenance of the transformed state may also depend on the continued expression of some cellular gene or genes. Thus, cells transformed by wild SV40 were selected that behave as typical transformants at 32°C but exhibit normal behavior at 39°C. Since, in this case, a mutant virus was not involved, the phenomenon is evidently due to a cellular function, the identification of which is of considerable interest.

Novel methods for stimulating the synthesis of C type viruses have forcefully confirmed the ubiquity of these agents in nature. Cells which, even by the most sensitive immunological tests, lacked any vestige of an RNA tumor virus, could be stimulated by BudR or other means to exhibit and replicate C type viruses,

lending support to the various theories regarding the cryptic residence of oncogenic information in apparently normal, healthy cells.

Investigations of cell membranes continue apace. Two developments are of unusual interest. One concerns the role that the carbohydrate fucose plays. Cells transformed by a mutant RNA virus (T5) are "normal" at 41°C but have the transformed phenotype at 35°C. The fucose moiety of the plasma membrane glycoproteins reflected the reversible phenotype, increasing at 35°C and decreasing at 41°C. It thus appears, in contrast to other carbohydrates, that fucose may be particularly involved in membrane changes associated with transformation.

An unusual report relating to membranes concerns the presence of a species of DNA in the plasma membrane of human lymphocytes. This DNA, representing 0.5 percent of the total DNA of the cell, has the properties of nuclear DNA. It appears to be synthesized in the nucleus and transported to the cytoplasm. The significance of this RNA and its function remains to be discovered.

It is gratifying to note the increasing number of investigations concerned with reversal of neoplastic behavior of cells, and further explorations regarding the relationship of this to differentiation. Prostaglandins may now be added to BudR (bromodexyuridine) DMSO, (dimethyl sulfoxide) and cyclic AMP (cAMP) as substances which influence differentiation and induce reversal of neoplastic behavior of cells. Striking synergism between cAMP and steroid hormones has also been reported. It has been suggested that the activity of cAMP is basic to these manifestations. Clearly, the mechanisms of these effects require intensive investigation. It is again suggested that a screening program to synthesize or isolate from nature new types of agents to affect differentiation and/or reversal of the malignant phenotype would be a worthwhile endeavor.

Future Outlook

From the above consideration, the direction that some areas of Cancer Biology will take is apparent. Not specifically mentioned is the continued and intensive investigations of cellular regulatory mechanisms, e.g., the regulation of gene expression and the control of cell division, and the further exploration of somatic cell genetics through cell fusion and hybridization coupled with a variety of new techniques to identify chromosomes and genes of animal cells.

A facet of cancer research that is in dire need of stimulation is metastasis. Means should be sought to encourage high quality effort in this rather frustrating and, so far, relatively unrewarding area of investigation. The recent suggestion of the beneficial effects of anticoagulants in reducing metastatic lesions in experimental animals and in man should provoke activity in this field. But more direct action might be needed. Perhaps a task force should be formed specifically to deal with this subject.

Administrative

A suggestion with respect to funding of approved regular research projects is offered. In order to spread research funds further, it is proposed to apportion levels of funding in accordance with the priority of the project. For example, the top 20 percent of approved projects might receive full recommended funding, the next 20 percent, 75 percent of the recommended levels, the next 20 percent, 50 percent of the recommended levels, and so on. Hopefully, both research and the research community would benefit from such a system.

CANCER EPIDEMIOLOGY PROGRAM

The extramural grants program in Cancer Epidemiology encompasses two main subprograms, epidemiology and biometry, and two minor subprograms, one in the behavioral sciences and the other, for administrative purposes, in biomedical communications.

Although representative research in cancer epidemiology has aimed traditionally to signal the urgency of a cancer problem and to generate hypotheses about the causes or the course of cancer for systematic controlled investigation in the laboratory or clinic, the present emphasis is on the verification of the generality in human populations of hypotheses evolving from the laboratory and the clinic. The epidemiologist finds and analyzes the frequency distributions of cancer of different forms, processes, and stages in selected sites of the body. He particularly studies how changes in these distributions relate to different forms, degrees and interactions of personal and environmental characteristics. Examples of personal characteristics include other pathologic states and physiologic factors. Examples of environmental characteristics include geographic, atmospheric, chemical, viral, economic, and social factors.

Representative research in cancer biometry aims to contribute to the design of convincing investigations not only in epidemiology but also in the laboratory and the clinic. Involved are the measurement of the different forms, processes, and stages of cancer at different sites of the body, the measurement of the personal and environmental characteristics that may bear antecedent, concurrent or consequent relationships to the various forms and processes of cancer, the modification of existing mathematical and statistical models, and the invention of new ones for the prediction of the course of cancer and for the convincing design and analysis of research on cancer problems and, finally, the application of computer technology to the design, conduct, and analysis of cancer research.

Funds from fiscal 1972 supported a marked growth of this program over fiscal 1971. The number of grants increased from 22 to 28 (27 percent), and the number of dollars from $1.4 million to $2.6 million (86 percent). In some respects the pattern of support has changed. Last year almost twice as many dollars went into program centers as into research projects; this year, however, slightly more money went into research projects (48 percent) than into program centers (46 percent). This change seems to come from an increase in the number of relatively new investigators who are becoming interested in cancer epidemiology and whose applications are successful at initial review for scientific merit. The dominance of the subprogram in epidemiology over the subprogram in biometry remains as in the past (73 percent to 22 percent).

Unchanged, too, is the pattern of support defined by the targets of cancer epidemiology. The major emphases continue to be on cancer of the breast, of the cervix, and of the blood and lymph. The plan is to continue to encourage applications with these emphases but to stimulate more applications with emphases on cancer of the colon and rectum, of the bladder, and of the prostate. From the viewpoint of antecedent conditions, emphasis continues

on demographic, viral and endocrinal factors, but applications are now being received which focus on chemical carcinogens, particularly in the atmosphere, and on social factors.

As promised in the annual report of last year, the Program Analysis and Reporting Section, Extramural Activities, has identified the extent to which research in cancer epidemiology, cancer biometry, and the behavioral aspects of cancer has been attached to projects, particularly program centers, supported by other NCI extramural grant programs. Forty-six grants were so identified. Twenty-three clinical cancer training grants included tumor registries, and two, studies of the behavioral aspects of cancer. Eighteen other grants included epidemiology; three, biometry; five, the behavioral aspects of cancer. It was estimated that, exclusive of the clinical cancer training grants, grants outside the Cancer Epidemiology Program as here defined contributed $640 thousand in support of epidemiology, $78 thousand in support of biometry and $28 thousand in support of the behavioral aspects of cancer.

The graduate research training grant program in Cancer Epidemiology has failed to match the growth in the research program. The number of grants is the same (3). Yet these three grants are productive. Two grants have been in effect only a little over two years, but each has several students whose doctoral theses are on problems of cancer. The one grant which has been in effect for about 10 years has produced, on the average, 2.2 graduates a year who are currently active in cancer epidemiology. It seems clear, then, that the release of more funds for graduate research training projects in Cancer Epidemiology can enhance the program. In addition, efforts will be made to invent new ways of producing cancer epidemiologists.

The workscope and functions of the Pharmacology Program relate primarily to the scientific and administrative supervision of preclinical research supported by federal grant funds. The program objective is to provide encouragement and financial support to outstanding scientists for research toward development of effective chemotherapeutic management of cancer. The systematic search for novel chemotherapeutic agents involves a coordinated effort of research in diverse scientific disciplines.

The Pharmacology Program area currently supports research and training in four primary categories based on successive stages of drug development for the treatment of cancer. Each category is interdependent upon and an integral segment of a total drug evaluation scheme. This includes (1) Synthesis and Isolation of new chemical compounds and pure principal constituents or well-defined products from natural sources; (2) Preclinical Drug Evaluation through screening tests, experimental therapeutics, dosage regimen, and toxicological studies; (3) Mechanisms of Drug Action, physiological disposition and related pharmacological studies and (4) Clinical Pharmacology and preliminary pharmacological evaluation in selected clinical investigations.

During fiscal year 1972, the Pharmacology Program supported 277 individual research projects with $12.46 million. Three program projects for research on a broad and flexible basis at departmental or interdepartmental levels involved an additional $1.39 million. Training programs for support of potential research investigators through graduate training grants, research career programs, and fellowship awards amounted to $2.02 million. The total fiscal commitment in the Pharmacology Program area was approximately $15.87 million. Research accomplishments and new scientific developments were reviewed on a continuing basis through surveys of progress reports and published literature resulting from grant supported research. Following is a summary of the research content and the fiscal apportionment within the principal program categories of the program.

1. Synthesis and Isolation of new anti-cancer agents: (145 grants, $5.96 million, 38 percent) Thirty-four projects involved isolation of potential anti-cancer agents from natural sources of microbial fermentation, plant and animal origins, and 111 projects were devoted to syntheses of new types of chemical compounds or analogs of nucleic acids, amino acids, proteins, pteridenes, terpenes alkaloids, organo-metallic and heterocyclic structures. Research on practical laboratory synthesis of analogs of adenosine-3'-5'-cyclic monophosphate (cyclic AMP) and isolation efforts from natural animal and plant sources have received increased emphasis in the current program.

2. Preclinical Drug Evaluation: (35 grants, $1.64 million, 10 percent) These projects related to evaluation of antitumor activity, experimental therapeutics and preclinical toxicology. Grant support of investigations for development or improvement of screening techniques for anti-cancer activity involved 24 projects. Principal effort in this area of drug evaluation is also conducted under projects supported by the National Cancer Institute's contracts program in the chemotherapy area.

3. Mechanisms of Drug Action: (91 grants, $4.30 million, 27 percent) Research on mode of action of known and potential anti-cancer drugs included enzymes, steroid compounds, folic acid and other antimetabolites, alkylating agents and nucleic acid analogs. The total effort in this category increased by approximately 30 percent during the current year.

4. Clinical Pharmacology: (6 grants, $0.56 million, 3 percent) Investigations of pharmacological and preliminary therapeutic aspects of new drugs were conducted through selected clinical trials. Some projects involve several phases of drug evaluation of new cancer chemotherapeutic agents. Selective toxicity and counteractive measures for prevention or supportive therapy of patients are important considerations in clinical pharmacology during establishment of safe and effective dosage schedules for extended clinical trials.

5. Research Program Project Grants: (3 grants, $1.39 million, 9 percent) These grants provide flexible support, usually directed toward solution of a range of chemotherapeutic problems within a broad category and having a central research focus rather than a specific single objective. Two grants provided support to departments of pharmacology and medicine. One project involved a multidisciplinary approach to the development of new drugs.

6. Research Training: (55 grants, $2.02 million, 13 percent) The purpose of research training grants is to encourage and to extend training of selected individuals preparing for research and academic careers in basic preclinical, clinical, and other scientific disciplines related to pharmacology. During the current fiscal year the overall distribution of support was as follows: Graduate Training Grants: 12 grants, $1.4 million; Research Career Programs: 12 grants, $0.34 million; and Fellowships: 31 awards, $0.28 million.

Recent Accomplishments and Leads for Future Research

1. Synthesis of analogs of cyclic AMP: Recently acquired data support the hypothesis that adenosine-3'-5'-cyclic monophosphate (cAMP) may act as an intracellular "second messenger" and as a mediator of hormone action. Growth of malignant cells in vitro responds to treatment with cyclic AMP. Permeability problems due to low lipid solubility and inactivation by cyclic phosphate diesterase within the cell prevent manifestation of activity in vivo. A systematic search is in progress for new cAMP analogs with improved physicochemical and biochemical properties. Preparation of dibutyryl derivatives and sulfur and selenium analogs of cAMP will be prepared to test for potential therapeutic activity against malignant growth. (CA 12591)

2. Maytansine, an active tumor inhibitor: An African plant, Maytanus ovatus yielded a new type of ansa macrolide, a class of compounds that includes rifamycins and streptovaricins. The compound is a potent inhibitor of a variety of tumors. Studies of Maytansine may help understand the chemical bases of biological activity of the other ansa compounds. The structure and functional groups are consistent with the "selective alkylation" theory proposed and developed as a common theme for explaining the inhibitory action of many natural products. (CA 11718)

3. Aberrant tRNA methylase capacity of neoplastic tissue: Investigations on natural inhibitors of tRNA methylases and alterations in nucleic acid of tumor cells revealed differences in tRNA methylase capacity of neoplastic tissue, presence of hypermethylated tRNA or altered tRNA in tumor tissue. Normal adult organ tissues contain natural inhibitor of tRNA methylase. The free enzyme without the inhibitor imparts greater capacity for methylation and proliferation. The inhibitor has been isolated into two components- a large protein with molecular weight of 100,000 and a smaller peptide of molecular weight 1000. The larger component was absent in growing embryonic or tumor tissues studied. These observations represent the first finding of a qualitative effect of a hormone. (CA 11286)

4. Deoxynucleosides as chemotherapeutic agents: Purine analog nucleotides selectively inhibit the chain initiation and chain termination at specific sites by RNA polymerase, whereas chain elongation in the presence of analogs remains indistinguishable from normal. These findings suggested that careful use of analogs should permit selective inhibition of transcription of specific regions of the genome or of the synthesis of specific classes of RNA molecules. This expectation was confirmed in several systems in vivo: for example, one adenosine analog (5-bromotubercidin) inhibits the expression of only very few (or even a single) late function of vaccinia virus. Another striking example is that of Rous sarcoma virus, whose RNA genome is known to be synthesized in a DNA-dependent reaction, as is the RNA of the host cell. Two analogs (toyocamycin and 5-fluorouridine) have now been found to inhibit selectively the transcription of cell-specific regions of the DNA genome without inhibiting the synthesis of virus-specific RNA. (CA 8290)

5. Cell cycle kinetics and therapy: An understanding of the role of cell cycle kinetics to cancer chemotherapy and the relatively widespread use of multiple mixtures of drugs have resulted in major advances in the management of acute leukemia. Knowledge of the biochemical mechanism of action of an effective chemotherapeutic agent and the pharmacologic factors influencing its action allows the utilization of this information to (a) tailor the magnitude and duration of therapy to the achievement of a particular metabolic event in neoplastic cells, (b) serve as a predictive test for cell sensitivity, and (c) design rational combination treatments. Additional gains in the treatment of acute leukemia would result from the widespread utilization of the principles of these scientific disciplines. (CA 2817)

Administration

An analysis of the research grants in the Pharmacology Program area has been completed. A systematic outline of categories and subcategories was developed in terms of objectives and goals of the National Cancer Institute. Each grant was assigned to an appropriate category. The scope and relative emphasis in individual program areas may be adjusted as necessary and as new developments occur in cancer research and therapy. Increased emphasis is placed on new sources of material and origins of potential chemotherapeutic agents, e.g., marine animals, insects, and plant sources. Greater effort is devoted to

specific areas of pharmacological research especially in investigations on mechanisms of drug actions. Follow-up studies on important findings and concepts in pharmacology related to interactions between cell constituents and chemical entities will be continued.

It is anticipated that the overall level and direction of research effort in the basic components of this program will continue to be maintained at approximately the present level. Because of the supplemental increase in the current fiscal appropriations for the national cancer effort, the total level of research support in the Pharmacology Program area increased by approximately 40 percent over that of the previous year. The policy regarding negotiated reductions of all awards was terminated. It was possible also, to lower the payment cut-off to approximately 60 percent of all recommended grants. Appropriate adjustments of program priorities have been maintained within the stipulated budget through careful assessment of the needs for research in individual component categories.

Future Plans

New projects in research and training may be initiated in the medicinal chemistry and pharmacology areas if adequate research grant funds remain available. Projected new programs include: (1) Cancer Pharmacology Centers: Development of pharmacology program centers involving collaborative research effort in preclinical and clinical pharmacology. (2) Intensified research effort in isolation, evaluation and synthesis of natural products, and laboratory syntheses of rare compounds. (3) Pharmacological and biochemical studies on specific neoplastic entities (e.g. melanomas, neurologic tumors, adrenal tumors. (4) Development of systems to predict effects of chemotherapeutic agents in man by prior testing either by "in vitro" procedures or by transplantation of excised human tumors into conditioned laboratory animals. (5) Research for development of new instrumentation and techniques. (6) Research on enzymatic patterns and requirements of normal and neoplastic cells; a search for specific metabolic requirements for development of rational therapeutic approaches.

CLINICAL INVESTIGATIONS BRANCH

Since its establishment in June 1968, the Clinical Investigations Branch has developed from one having concern almost exclusively with the chemotherapy cooperative groups to become that segment of Extramural Activities, NCI, which brings together all the therapeutically-oriented, grant-supported programs into a single administrative component in geographical proximity.

The current programs of the Branch are:

Cooperative Clinical Investigation
Leukemia (Platelet)
Protected Environment
Radiation, including
  Radiotherapy
  Radiation Biology, and Physics
Chemotherapy
Immunology
Surgery

Detailed reports for each of these program areas follow.

In addition to the administrative and scientific guidance components of these various programs, the Branch provides staff support for the Cancer Clinical Investigation Review Committee, one of the five NCI primary review groups. This Review Committee has the responsibility for both programmatic and grant application review of the first three programs listed above. The Chief of the Branch, in addition to his other duties, functions as Executive Secretary of the Cancer Clinical Investigation Review Committee.

During the past year, because of the progressively increasing scientific breadth and number of grants in the Radiation Program, a more basically oriented program for Radiation Biology and Physics has been developed with a separate Program Director. The Director for this Program is a radiation physicist who will provide a desirable chronological continuity to supplement the annual change of Radiotherapy Program Director. That this annual change has been so successful over the past three years is because of the effective work of members of the radiation therapy community who have filled this post during a sabbatical year from their own universities.

The position of Surgery Program Director is presently vacant due to a shift of the former Program Director's activities into the burgeoning Cancer Centers Program. Active recruitment for a successor is under way.

The Chemotherapy Program, for which a Program Director was found just prior to the beginning of the year covered by this report, has provided an important focal point for continuing coordinative review of all grant-supported chemotherapy research in man. The Director of this Program has been of great assistance in the scientific evaluation of the Cooperative Clinical Investigation Program, as well as providing significantly increased coordination and communication between these areas of Extramural Activities and the therapeutically-oriented activities of the Chemotherapy Program segment.

The geographic proximity of the various Program Directors within the Branch has led to increasingly effective communication. The subsequent beneficial mutual input among the programs has progressed gratifyingly. As the extent of all the programs continues to increase, the need for a comparable increase in nonprofessional staff (both at and above the secretary level) in each program becomes greater. This support is needed to relieve the professional scientist-administrator of the many distracting administrative facets of program guidance which truly do not require his unique expertise. It would also provide staff support for the program analysis so sorely needed in all program areas. The usefulness and importance of this type of staff support has already been amply demonstrated for the Cooperative Clinical Investigation Program. It is hoped that this will be extended to the other programs of the Branch during fiscal year 1973.

Constituents of the Program in Fiscal Year 1972

The previous report discussed in some detail the addition of several cooperative groups to the program and the discontinuance of others. There have been no additions or deletions during fiscal year 1972. The currently active groups are:

Acute Leukemia Group B
Children's Cancer Study Group A
Southeastern Cancer Chemotherapy Study Group
Southwest Cancer Chemotherapy Study Group
Western Cancer Study Group
Eastern Cooperative Oncology Group
Central Oncology Group
Polycythemia Vera Study Group
National Wilms' Tumor Study Group
Cooperative Breast Cancer Group
Primary Breast Cancer Group (NSABP)
Gynecologic Oncology Group
Surgical Adjuvant Renal Cell Carcinoma Group
Veterans Administration Urological Research Group
Radiation Therapy Oncology Group
Radiotherapy Prostatic Cancer Group
Radiotherapy Hodgkin's Disease Group
Radiotherapy-Chemotherapy Head and Neck Cancer Group
Preoperative Irradiation Lung Cancer Group.

With the participation of more than 1,200 investigators in over 300 institutions throughout this country and in several countries abroad, upwards of 18,000 patients have been studied during fiscal year 1972 through the utilization of more than 400 protocols. The total grant support of this program for fiscal year 1972 is $10.2 million.

However, the continuing annual increase in fiscal support, numbers of participants, and numbers of patients studied on group protocols indicates only part of the quantitative nature of the changes occurring in this Program. In fiscal year 1969, the first year of this CIB Program, 14 of the present 19 groups were active with a total of 101 grants. This past year, there were 166 grants for the 19 active groups. There has been an increase in active protocols from 230 in fiscal year 1971 to 400 in fiscal year 1972. As about one-third of this total number of protocols represents closing of older studies and activation of new ones, the continuous process of protocol review and collation within the CIB is an ongoing and ever increasing task.

Group reporting requirements have developed so that more than 45 separate sets of detailed minutes of various group meetings were received, reviewed, and distributed by the CIB in fiscal year 1972. These group reports provide the necessary current information for proper review of both scientific and administrative aspects of group activities.

The qualitative nature of the Program's changes is threefold. First are the changes in the disease areas studied. This Program has long been considered by many as being predominantly involved with the evaluation of various chemotherapeutic agents for the treatment of acute leukemia. Although still a significant component, the shift towards other areas is indicated by the facts that only the first five of the 19 groups listed above have protocols for acute leukemia, and four of the five have less than half their protocols for this disease!

Phase III surgical adjuvant studies have been initiated by three groups never before involved in such efforts, an indication of the effects of the new disease-oriented subcommittee structure of most of the groups.

The second qualitative change is with regard to the type of protocols being initiated. In the first 10 months of fiscal year 1972, 63 new chemotherapy protocols were activated. Only seven of these were Phase I dose-seeking studies of the toxicity of new agents, and 27 were Phase II studies testing for anticancer activity in man. Twenty-nine studies (46 percent) were large and complex Phase III prospective randomized studies comparing new treatment regimens with the present standard method. This changing pattern of Phase I-II-III studies is a clear indication that the 'drug testing' aspect of the Program is waning and that solid therapeutic progress is the major goal.

The third qualitative change is the addition of more extensive laboratory investigations to many of the studies which were formerly mostly clinical. Several groups are initiating serial assessments of the immunologic capacity of each patient. Review and correlation of histopathologic material from protocol patients is receiving a gratifyingly increased attention. Three groups have developed Pharmacology Support Centers which will undertake in depth evaluations of many pharmacokinetic and pharmacologic parameters previously studied in only very small groups of patients, if at all.

These qualitative changes are all indicative of a growing sophistication of the work of the participants in the program, and will obviously be vigorously fostered in the ensuing year.

Meetings and Symposia

Two sets of national meetings have been developed in connection with the Program. The first is a biannual two-day working conference of the statisticians and data processing supervisors from the various cooperative groups. These highly productive sessions are an outgrowth of the increased concern for this aspect of cooperative clinical studies noted in last year's report. The success of these meetings is in large part due to the organizational and planning efforts of the Program Administrator, Mr. Clare White, working closely with the Statistics and Data Processing Subcommittee of the Cancer Clinical Investigation Review Committee and with the group statisticians themselves. The agendas have been an effective blend of the presentation and discussion of new biostatistical approaches, demonstrations of modus operandi by representatives of the host cooperative group, and workshops on topics of mutual interest and concern.

The second set of meetings to be reported are the continuing symposia and workshops sponsored jointly by the CIB and the CCIRC. The primary organizational efforts have been carried out by CIB staff, while agenda development has been done by designated CCIRC members. Proceedings of the first such symposium, Prediction of Response in Cancer Therapy, held in February 1970, were recently published as Monograph No. 34 of the Journal of the National Cancer Institute. The proceedings of the Symposium on Statistical Aspects of Protocol Design, held December 9-10, 1970, have been published and distributed to all the participants, to all principal investigators in the Program, and to many medical schools and teaching hospitals here and abroad.

A Workshop Symposium on Cell Kinetics, Cell Biology, and Solid Tumor Therapy was held March 22-24, 1971. Three concurrent workshops were a part of this working meeting. Although it has not been thought advisable to seek publication of the reports of the workshops on colorectal cancer and on lung cancer, the discussions and conclusions reached during these sessions have had widespread effect among the cooperative groups. The proceedings of the workshop on bladder cancer will be published shortly in the Journal of Urology.

The clinically oriented Symposium on Diagnosis and Treatment of Hodgkin's Disease held October 7-9, 1971, was attended by over 500 members of the medical profession, including participants from Mexico and Canada as well as the United States. Most of the persons attending were not active participants of the Cooperative Clinical Cancer Program so that the educational impact of this meeting was widespread. Editing of the proceedings is essentially complete and they will be published in the near future in the Archives of Internal Medicine.

A two-day Symposium Workshop in Criteria of Response to Therapy was held in January 1972. There was representation from every one of the cooperative groups in the program, and the discussion was spirited. Original manuscript copies or stenotypic transcriptions of both the presented papers and the concomitant workshop sessions are being edited, and it is anticipated that these proceedings will be published in Cancer in the near future.

Plans are under way for future symposia workshops, tentatively in the areas of pharmacology of chemotherapeutic agents, interrelations between radiation therapy and chemotherapy, and metabolic effects of neoplastic disease. Plans are also under way for a clinically oriented educational symposium on multiple myeloma.

Advances in Therapy Resulting From Program Activities

Several significant advances in the management of patients with neoplastic disease have become apparent from the studies carried out under the auspices of this program during the past year.

The importance of long-term maintenance therapy, following remission induction in advanced cases of Hodgkin's Disease, has been clearly demonstrated by two separate cooperative groups. Such patients, formerly having a relatively dismal outlook, now have about a 50 percent chance of developing a complete remission and remaining disease-free for many months thereafter. As these studies

are still underway, it is expected that a significant number of these patients will continue to remain disease-free for some years to come.

The progressive clarification of prognostically significant subclassifications of the lymphomas has led to identification of certain of these subclassifications which respond more favorably to vigorous combination therapy than do others. In the studies of one of the cooperative groups, two combination drug regimens of comparable effectiveness in Hodgkin's Disease were found to have important differences between the regimens when used for the treatment of reticulum cell sarcoma and of lymphosarcoma. This continuing breakdown of prognostic characteristics holds considerable promise for use of a more selective approach to each of these various subentities.

In a prospective randomized study comparing a widely acclaimed five-drug regimen for treatment of metastatic breast cancer to a simpler and less toxic three-drug regimen, one of the cooperative groups has clearly demonstrated the lack of superiority of the more rigorous and toxic five-drug regimen. Another group in a similarly prospective randomized study has shown that palliation of brain metastasis from various types of primary tumors can be equally well achieved with a course of radiation therapy less than half as prolonged and intensive as that initially thought necessary. Both of these results are of importance in that the therapeutic gain to the patients can be achieved with less costly and toxic regimens than previously considered necessary.

Considerations for Future Years

Three new groups are under review by the Cancer Clinical Investigation Review Committee as potential additions to the program. The first of these is a group of diagnostic radiotherapists whose studies will provide clearer assessment of the role of early radiographic diagnosis of primary breast cancer. They also will seek to identify characteristics identifying a group of women at unusually high risk of developing breast cancer, so that the currently available radiographic screening techniques may be applied more appropriately. Two dermatologic groups have been initiated. One of these groups is developing a multifaceted study of mycosis fungoides, a neoplastic entity presently receiving little serious investigation. The second group is devoting its activities toward the study of malignant melanoma in its early stages. They also have a multifaceted scope of investigation, with the goal of developing a simple and useful criterion that will permit earlier diagnosis of invasive melanoma. They will also assess the effectiveness of such earlier diagnosis and treatment on the survival of patients with this disease, an entity currently very resistant to successful therapy in its later stages.

The progress of the Pediatric Intergroup Liaison Committee has been gratifying. This committee will remain operational for the development of protocols for intergroup evaluation of additional uncommon pediatric solid tumors. Case accrual to their joint studies of Ewing's tumor and of rhabdomyosarcoma will be well underway by the end of 1972. In association with this group, a pediatric solid tumor pathology panel has been formed and will be seeking grant support in early fiscal year 1973.

Reorganization of the operations of the Cancer Clinical Investigation Review Committee has been accomplished with increased effectiveness in the joint efforts of the Branch and the Review Committee. Many aspects of the increased activities of the Review Committee, although enthusiastically desired by both the Committee and the CIB, have not yet been implemented because of lack of staff personnel in the CIB. In the face of the steadily increasing activities of the program referred to above in several areas, CIB staff available for this program has remained constant during the past two fiscal years. Additional personnel will be required if the increase in sophistication and effectiveness of the studies in this program is to be maintained.

LEUKEMIA (PLATELET) PROGRAM

In the past two years the Cancer Clinical Investigation Review Committee and the Clinical Investigations Branch have carried out a thorough review of the current situation regarding platelet transfusions for thrombocytopenia as a consequence of acute leukemia or its treatment. A new set of guidelines for the program was developed, provided to all ongoing investigators and many new interested ones, and revised grant applications from the previous participants reviewed. Several new investigators also have submitted applications for support from this program and they have been reviewed. Recently, techniques have become available for isolating leukocytes in quantity to use for transfusion in leukocytopenia states associated with malignant disease and its treatment. Grant applications for support of studies in leukocyte transfusions have been awarded in this program during the past year.

There are now 21 institutions receiving support for studies of platelet and/or leukocyte transfusions as supportive measures during chemotherapy for acute leukemia. These institutions utilize approximately 70,000 units of platelets annually in their studies. An ongoing assessment of many of the factors involved in the success of platelet transfusions is being carried out through this program. Two institutions are performing prospective randomized studies to develop clear indications for the use of platelet transfusions and to delineate their efficacy when used under varying circumstances.

Five of the institutions have active programs for the study of leukocyte transfusion, assessing different techniques for leukocyte procurement and storage. In one institution a prospective randomized study of the clinical efficacy of leukocyte transfusions has been initiated.

It is anticipated that the indications for the use of these two biological materials with a limited supply will result in more specific and effective utilization of these unique biologic materials.

PROTECTED ENVIRONMENT PROGRAM

Beginning in fiscal year 1966, grant support has been provided for the study of intensive reverse-isolation techniques designed to prevent serious infections in patients undergoing vigorous chemotherapy for neoplastic disease. These techniques have involved the use of either the Life Island or the Laminar Air Flow units in which everything coming in contact with the patient has previously been sterilized. These units require major personnel support of all categories of professional and paramedical personnel and also require extensive bacteriologic monitoring.

The intensive review of this program and of the participants in it carried out during the previous year has resulted in the discontinuance of two of the four institutions' programs formerly active. At both institutions the problems were those of ineffective local institutional support rather than lack of interest or competence of the investigators to pursue the programs.

In the two currently supported protected environment studies, urgently needed prospective randomized studies are underway to assess the usefulness of this costly support measure in the treatment of acute leukemia. The evidence available to date strongly suggests that a reduction in severity and incidence of infection may be achieved utilizing these techniques. However, it is by no means clear at present that an increase in patient survival is a concomitant of the decreased infection. The randomized studies underway in this program should provide data soon to answer this question. The upsurge of specialized cancer unit construction throughout the country often includes plans to construct protected environment units, the usefulness of which is still in doubt. A positive or negative answer to this question will affect the design and cost of many of these special cancer units currently being built.

The Radiation Programs are concerned with research and training in clinical radiotherapy, radiation biology, and radiation physics. Because of the unusual scientific breadth of the Programs, a second program director has been added, for Radiation Biology and Physics, to complement the Program Director for Radiotherapy. Program activities continue to be supported by means of traditional research grants, exploratory grants, research center grants, graduate training grants, and grants for cooperative clinical studies. During this year it has been possible to continue funding traditional grants and research center grants at a high level; however, it has not been possible to fund training and exploratory grants at a comparable level. Efforts of the program directors have been focused primarily in two areas: (1) exploratory projects which continue to be valuable in planning for future development of clinical radiation research centers; and (2) high LET radiations, which offer some hope of significant breakthroughs in radiation therapy.

Radiation Therapy Clinical Research Centers

This type of center continues to play a very important role in cancer management in many major clinical facilities throughout the country. Since it is well recognized that radiation therapy is essential in the management of cancer patients there is continuing interest in developing new radiation centers, particularly in medical schools. This facet of the Radiation Programs continues to grow, although somewhat slowly. At present twelve of these centers are being funded for a total of over $5.0 million. During this year six new exploratory projects have been approved that could lead to expansion of existing radiation centers or the creation of new ones. Already a trend is detectable in that at some institutions ongoing radiation research centers are becoming the nucleus for the creation of comprehensive multidisciplinary cancer centers. It appears likely that in the future exploratory projects that originate in radiation therapy departments would be concerned with planning the development of this type of facility as a stepping stone to ultimate development of a comprehensive cancer center. This is a logical evolution since radiation therapists as a group are the physicians most closely identified with cancer in any major medical center.

Training in Radiation Therapy, Radiation Physics, and Radiation Biology

Through past efforts of the National Cancer Institute the shortage of trained radiation therapists in the country has lessened. Following a policy of special emphasis of increased training in this field 36 institutions are receiving support by means of training grants for a total dollar amount just over $3.6 million. Through these grants approximately one half of all physicians receiving training in radiotherapy at the present time are being supported by the National Cancer Institute. Despite these efforts there continues to be a critical shortage of radiation therapists as shown by a survey conducted by the Committee for Radiation Therapy Studies. It is unfortunate that most ongoing training programs have not sought support from sources other than the National Cancer Institute to continue operation. In view of the limited monies available for training such other sources of support must be sought to allow more new training programs.

A companion manpower problem is the need for training radiation physicists to provide the support required by radiation therapists in carrying out optimal forms of treatment. The American Association of Physicists in Medicine has shown great interest in this problem and grant proposals for training in medical or radiological physics can be anticipated in the future.

Cooperative Clinical Studies

The participation of radiation therapists in cooperative clinical studies continues to increase in a very gratifying fashion. The first such study initiated by the Committee for Radiation Therapy Studies, dealing with preoperative irradiation in lung cancer, has been completed. Other studies dealing with Hodgkin's disease and head and neck cancer are near to completing accession of patients into the studies.

More recently, 25 institutions agreed to form the Radiation Therapy Oncology Group (RTOG) to carry out cooperative clinical trials. This group has been reviewed favorably by the Cancer Clinical Investigation Review Committee and the National Advisory Cancer Council and is now receiving funds for its activities. At the present time this group is involved in projects studying irradiation of brain metastasis, split course of irradiation, and the effectiveness of hyperbaric oxygen and oxygen breathing in conjunction with radiation therapy for a variety of malignancies.

The creation of the RTOG is very encouraging since it will likely lead to a more meaningful dialogue with other groups already participating in cooperative clinical trials under the aegis of the Clinical Investigations Branch. It is expected that the RTOG will come forth with additional protocols and, also, will help some other cooperative clinical groups in the preparation of protocols involving radiation therapy.

Research Grants

This category covers generally unsolicited research grants. At present about 60 grants of this type are being supported through this program. The total dollar amount is just over $3.0 million. The scientific content of this category is broad spectrum, from the very basic to the quite applied. It cuts across the disciplines of physics, chemistry and biology. Basic studies include investigations into the molecular basis for radiation lethality and biochemical changes caused by radiation inactivation. Applied studies include computer analyses of tumor roentgenograms and optimizing automated radiation treatment planning. Physics studies include proton energy loss measurements. Chemistry studies include photochemistry of biologically important bases. Biology studies include RNA synthesis in mammalian cells after irradiation. The overall goal of this funding mechanism in the radiation program is to broaden the understanding of the interaction of radiation with biological material and to increase the impact of such information on the cure and prevention of cancer.

Prospects for the Future

A most critical need of the national attack on cancer is the creation of multidisciplinary cancer centers concerned with research, training, and other activities. Already existing radiation research centers are likely to play a leading role in this regard by providing a nuclei around which

multidisciplinary cancer centers can be formed. Therefore, a primary goal of the Radiation Programs is to strengthen existing radiation research centers and encourage the development of new ones especially in geographical areas lacking these fundamental components for the study and treatment of cancer. Exploratory grants will continue to prove useful as a tool for creating new radiation research centers and for strengthening already existing ones.

Another important goal in the Radiation Programs over the next few years is the assessment of the role of high LET particle radiation in cancer therapy. There is already considerable experimental data suggesting that low oxygen tension renders mammalian cells less radiosensitive to the effects of X-ray or gamma radiation. Since most human tumors contain necrotic foci, it is very likely that at least part of the tumor population is hypoxic, which could account for many radiation failures. In fact it has been estimated that annually there are approximately 50,000 patients treated by radiation in whom the initial manifestation of recurring disease is at the site of the primary tumor. There is a growing consensus that many, if not most, of these local failures are due to the hypoxic tumor population which if overcome could yield dramatic improvements in cure rates. Excitement over high LET radiation is based on growing evidence that it may overcome the radioresistance of hypoxic tumor cells. A pilot study being carried out at Hammersmith Hospital in London appears to substantiate this for fast neutrons. In order to confirm these observations, if they are real, the National Cancer Institute is supporting several research projects in this country, using cyclotron-produced fast neutrons, that should lead to clinical trials in the near future. In addition, support is being provided for projects that are studying the feasibility of designing a deuteron-tritium generator for the production of neutrons that could provide a clinically suitable source of neutrons for cancer therapy.

Another form of high LET particle radiation which seems to offer similar biologic advantage is negative pi mesons. In collaboration with the Atomic Energy Commission the National Cancer Institute is funding the design and construction of a biomedical channel at the Los Alamos Meson Facility to study the possible clinical applications of this type of particle.

During fiscal year 1972 approximately $1.0 million is being spent on high LET radiation efforts and it is anticipated that during fiscal year 1973 over $2.0 million will be spent in this area.

The chemotherapy program (CH) is the newest program in the Clinical Investigations Branch, having been organized in April 1971. During its first fiscal year (1972), 15 traditional research grants and one training grant were funded and approved (funding for fiscal year 1972 = $477,697).

This program provides scientific direction for grant-supported research involving use of drug treatment for cancer patients. The four main subdivisions of the program and the progress made during the first year of activity for the chemotherapy program are as follows:

I. Clinical Therapeutics

Phase I, II, and III and adjuvant studies form the basis for this aspect. The most efficient means for carrying out such studies are the protocols of the clinical cooperative groups funded through the Clinical Investigations Branch. The Chemotherapy Program Director (CPD) has spent the majority of her time involved in the scientific aspects of these cooperative groups as follows:

A. Critical scientific appraisal of the protocols generated by the groups. All protocols are carefully reviewed by the CPD and the Chief of the Clinical Investigations Branch. The CPD communicates criticisms to the groups and aids the group investigators in up-grading their protocol studies.

B. Follow-up of the progress of group studies through careful analysis of group minutes and progress reports. The CPD also attends group meetings and communicates between meetings with the group study chairmen.

C. Through a search for gap areas, the CPD helps to generate ideas for new protocol studies.

D. Aids the Cancer Clinical Investigation Review Committee (CCIRC) in its exhaustive review of cooperative groups. The critique prepared by the CPD for each group protocol provides initial scientific evaluation for the CCIRC.

In addition to the studies of the cooperative groups, the CPD has attended several center site visits with special emphasis on those centers proposing chemotherapy studies. Thus far, there are no traditional grants approved in the area of Clinical Therapeutics.

II. Developmental Studies

This second area is the basis for development of new drug treatment programs. The Chemotherapy Program is involved with the ancillary studies of group investigators working in the Pharmacology Support Centers reviewed by the CCIRC. The traditional grants in progress include cellular kinetic studies

(especially with regard to treatment of cervical carcinoma), regulation of granulopoeisis, chromosomal analyses in certain malignant disorders, and development of the application of mass spectrometry for studies which may be able to determine specific responsiveness of tumors to certain drugs in vitro.

III. Drug Toxicity

Concern for the toxic effects of chemotherapeutic agents is a very important aspect of all clinical studies. The CPD carefully monitors all proposed and on-going cooperative group studies for such toxic events, and communicates information as is necessary to investigators engaged in similar endeavors.

The protection of normal tissue during drug treatment is an important area of the program which is being developed. Discussions with certain cooperative group chairmen have taken place and from these, we may be seeing development of new studies.

Coordination of studies regarding the supportive care necessary after vigorous anticancer drug therapy is facilitated by the CPD's involvement with the Leukemia (Platelet) Program and the Protected Environment Program of the Clinical Investigations Branch. Studies of bone marrow preservation, platelet transfusion and white blood cell defects leading to infection are being funded under the traditional research grants.

IV. Cancer Related Disorders

There are several important clinical problems which develop during chemotherapy in cancer patients and which may affect drug treatment. Though quite important, this is thus far the smallest aspect of the Chemotherapy Program with only one traditional grant currently being funded.

In summary, the Chemotherapy Program, though still in its early stages, is growing. New avenues have been explored this past fiscal year. As time goes on, additional progress should be noted especially in those areas mentioned above.

Fiscal year 1972 is the third year of the existence of the Immunology Program. This program has progressed from a budget of $5,285,600 in fiscal year 1970, through $5,287,570 in fiscal year 1971, to a total of $7,232,210 in fiscal year 1972. The fiscal year 1972 amount includes the support of 134 research grants totaling $4,729,369; five program project grants totaling $1,390,007; and 15 training grants totaling $1,112,834. The $1.9 million increase in fiscal year 1972 over 1971 has enabled us to expand modestly the Immunology Program. Much more is needed, however, to allow for diversification and for coverage of areas of tumor immunology that are not yet being adequately studied.

Tumor immunology continues to progress and to attract the interest of those concerned with problems of the diagnosis, prevention, and therapy of neoplasms. It is generally accepted that the cells of both virus-induced and carcinogen-induced tumors in experimental animals, as well as various tumors of unknown etiology in man, contain antigenic determinants against which the host mounts an immune response. This response may be manifested in various ways, and may be associated with both humoral and cell-mediated immunity. The current consensus is that cell-mediated immunity is probably of greater concern in tumor regression than is humoral immunity. Clarification of this issue awaits further study, however, since there are reports of a protective effect of serum antibody. Whether or not humoral factors play a role in defense against tumor growth, there are indications that they play a role in tumor regression by blocking the effective cell-mediated antibody. In addition, the detection of tumor specific humoral antibody might serve as a basis for a specific diagnostic test for certain tumors. Much more work is needed to exploit this possibility.

The current year has seen major advances in several areas of tumor immunology. Most important is the study in which it has been clearly shown that mice with growing tumors can be "cured" of the malignancy by the inoculation of tumor cells which have been treated with neurominidase. Whether or not this activity of neurominidase-treated cells can be increased by the use of other adjuvants, such as BCG, is under study. Another meritorious advance is the development of a highly sensitive and reproducible test for the measurement of carcino-embryonic antigen in human plasma. This test, capable of measuring in nanagram quantities, has already been pretested to the extent that it is ready for use in mass surveys. It can be performed on a minimum of one-tenth milliliter of serum and is so standardized and easily performed that one individual can perform 200 tests per day with great accuracy. Use of this radio-immuno assay may, within a year, settle the important question of the prognostic and diagnostic usefulness of this test in human cancer.

During the past year the Program Director has spent considerable time in the organization of the Immunology Program. An advisory committee consisting of D. Bernard Amos, M. D., Professor of Immunology, Duke University Medical Center; Martin H. Flax, M. D., Ph. D., Chairman, Department of Pathology, Tufts University School of Medicine; Ingegerd Hellstrom, M. D., Research Associate Professor of Microbiology, University of Washington School of Medicine; Hugh McDevitt, M. D., Associate Professor of Medicine, Chief, Division of Immunology, Stanford University Medical Center; Noel R. Rose, M. D., Ph. D., Professor and Director, The Center for Immunology, State University of New York at Buffalo;

and George Santos, M. D., Associate Professor, Department of Medicine, The Johns Hopkins University, School of Medicine,was formed early in the year and has met three times. Under primary consideration by this committee are the following:

I. Communication

A. To increase and to accelerate communication among individuals working in the area of tumor immunology.

B. To inform and to inspire highly regarded immunologists who are not yet working in tumor immunology to become interested in research in this area.

As a first step, the Immunology Program is sponsoring a meeting of all individuals who hold grants in the Tumor Immunology Program. This will be held in October at Wood's Hole and if successful, will be set up as an annual event. In addition, consideration has been given to the sponsorship of an annual symposium; the feasibility of setting this up under the aegis of the Gordon Conferences is under consideration. In addition, the program supports, by direct grant, tumor immunology symposia originating with other bodies.

II. Training

The committee is acutely aware of the limited number of trained tumor immunologists,including both the strictly research scientist and the research physician. To assess the extent of the need for training in this area, a questionnaire is being prepared to go out to the memberships of the following societies: American Association of Immunologists, American Association of Cancer Research, American Society of Clinical Oncology, Society of University Surgeons, American Society for Experimental Pathology, and the Transplantation Society to determine potential interest in this area as well as the number of individuals now on hand who are working in tumor immunology research or who could work in cancer therapy teams if rapid advances are made in this area and it is judged timely for immunotherapeutic trials in cancer hospitals to begin.

III. Research

The committee spent considerable time on the consideration of areas of immunological research as they apply to tumor immunology. Especially emphasized was the need for further research in the following:

A. Immunization with cells.

1. Methodology to increase cell-mediated antibody response to cellular antigens, including choice of adjuvants.

2. Description of response of host to cellular tumor antigens.

3. In vitro modification of the surface of cells to alter antigenicity.

B. Isolation and purification of tumor specific transplantation antigens.

C. Immune response--any approach to manipulation of the immune response to favor host survival.

1. Definition of immune status of tumor-bearing host.

2. Stimulation of immune response - specific - nonspecific, including preferential stimulation of cell-mediated in the absence of humoral immunity.

3. Effect of cancer chemotherapeutic drugs, hormones, irradiation, or combinations of these on the immune response.

4. Inhibitors of immune response.

5. Mechanisms of immunologic recognition.

6. Mediators of cellular immunity.

7. Genetics of immune response.

8. Isolation and separation of different kinds of circulating antibody and a definition of the role of each kind in protection and enhancement.

D. Determination of what immunological test or tests reliably correlate with the clinical status of the tumor-bearing patient.

E. Models for immunotherapy and for combined therapy.

F. Models for prevention by active or passive immunization with immune serum or cells.

G. Histochemistry of the tumor cell being rejected.

H. Conditions for culture of mouse lymphocytes.

The Immunology Program feels strongly that the grant-supported research project or program project emphasizing immunology can make substantive contributions to the overall Immunology Program of the National Cancer Program. With this conviction, attempts are being made to intensify and to diversify in an attempt to cover adequately all of the major areas of immunology bearing on problems of oncology. It is believed that this grant-supported Immunology Program, independent in concept and in execution, will dovetail nicely with immunology programs supported by other mechanisms, including National Cancer Institute intramural research, contract supported research, and research supported by other mechanisms. The achievement of this goal--to preserve a significant part of tumor immunology research funds for the investigator-instigated research proposal--will necessitate a significant increase in the funding of the Immunology Program.

CLINICAL CANCER TRAINING PROGRAM

Clinical Cancer Training grants are awarded to medical schools, dental schools, schools of osteopathy, specialized cancer institutions, principal affiliated hospitals of medical schools, and schools of public health. The purpose of these awards is to:

(1) encourage the planning and development of educational programs at undergraduate, graduate, and postgraduate levels, aimed at the ultimate achievement of optimal care of the cancer patient;

(2) enable trainees in the health professions to acquire basic knowledge of neoplastic disease and of preventive measures, and diagnostic and therapeutic skills and experience in optimal cancer care; and

(3) stimulate and expand efforts in cancer teaching and training which will be relevant to changing needs in medical and dental education, and in the delivery of health care services as they apply to the cancer patient.

In 1971, there were 105 active Clinical Cancer Training grants distributed among 71 medical schools, 26 dental schools, 2 hospitals, and 6 specialized cancer institutes at a cost of $5,700,000. The distribution of grantee institutions by state is as follows:

Alabama	2	New York	13
California	9	North Carolina	3
Colorado	2	Ohio	4
Connecticut	1	Oklahoma	1
District of Columbia	3	Oregon	1
Florida	1	Pennsylvania	7
Georgia	3	Puerto Rico	2
Hawaii	1	South Carolina	1
Illinois	5	South Dakota	1
Indiana	1	Tennessee	6
Iowa	1	Texas	3
Kentucky	4	Utah	1
Louisiana	2	Vermont	1
Maryland	3	Virginia	3
Massachusetts	5	West Virginia	1
Michigan	3	Wisconsin	1
Minnesota	2		
Mississippi	1		105
Missouri	4		
Nebraska	1		
New Hampshire	1		
New Jersey	1		

While the Clinical Cancer Training grants provide opportunities for enriching the cancer teaching and training experiences of all undergraduate and graduate

students in a grantee institution, stipends are also made available to selected trainees at various levels of their training. More than 394 trainees have been reported to date for fiscal year 1971. However, a total number of 600 is expected. The number of trainees reported since the inception of the program is 2,511.

Administrative:

Two training committees, one to consider only proposals from dental schools, were established in 1965 to provide technical assistance and advice on all applications, to make recommendations to the National Advisory Cancer Council and to the Director of the National Cancer Institute, and to maintain a continuing awareness of the needs for personnel and facilities in the field of cancer teaching.

The training committees have devoted considerable time to the development of techniques for the evaluation of proposals for training and for the assessment of ongoing Clinical Cancer Training Programs. New Guidelines formulated by each of the Committees were issued in March, 1971, in order to define more clearly the goals, organization, administrative structure and characteristics considered most appropriate for sound cancer teaching and training programs. The Program is continually evaluated through project site visits, for which lists of specific criteria have been formulated, and by review and analysis of progress reports from the training institutions.

Despite an administrative supplement, funds appropriated for fiscal year 1971 not only did not permit expansion of existing programs nor allow support of all new programs which were recommended for approval but required reductions in ongoing training. The plan to extend eligibility to additional teaching hospitals and encourage applications from new medical and dental schools has once again been deferred.

Liaison has been established and maintained with other Federal programs such as the Regional Medical Program, and within the National Cancer Institute, close liaison is maintained with all related programs.

GRADUATE RESEARCH TRAINING PROGRAM

Awards in support of graduate research training programs are made by the National Cancer Institute in order to assist qualified institutions to initiate or to continue training designed to encourage and aid medical and basic scientists to achieve productive careers in academic medicine. These programs provide a source of competent research manpower that is directed toward alleviating shortages of skilled personnel in areas with significant relevance to the cancer problem.

Training grant support, paralleling research grant support, is distributed among the areas of cancer biology, carcinogenesis, cancer epidemiology, cancer immunology, cancer pharmacology, and radiation (comprising radiation therapy, radiobiology, medical physics, and nuclear medicine). Although the Institute continues to be assigned applications which have not been programmed, most of the proposals are generated as a result of staff contact with departments and institutions that have demonstrated an ability to develop and carry on vigorous and effective cancer training in those areas where there is a continuing need for trained professional personnel for teaching and research. Both Extramural Staff and Advisory Personnel contribute to the identification of specific areas for which the need for increased training opportunities is manifest as well as to the assignment of priorities among the areas that are in competition for funds budgeted for training.

In addition to providing the initial review of applications, the Cancer Research Training Committee maintains a surveillance of the accomplishments of ongoing programs in order to assess the progress being made and to determine whether the training on each program continues to be focused on the cancer problem. The Committee has also expressed concern that existing stipend ceilings are below the amounts paid by many universities, from their own funds, to predoctoral and teaching and research fellows, and by many hospitals to interns and first year residents. This becomes an important factor in recruiting since a candidate is often reluctant to accept an appointment as a trainee if the stipend he would receive would be considerably below that being paid to teaching fellows and residents with comparable amounts of training and experience. The Committee believes that ceilings should be revised to permit payment of stipend amounts that are comparable with stipends paid from sources other than training grants.

Approximately $5,317,000 of fiscal year 1971 funds were awarded for support of 86 training programs. These funds were distributed among 57 institutions in 27 states and Puerto Rico. Training was provided for 550 stipendiaries as well as for a number of individuals who, while deriving considerable training benefits, received stipend support from a source other than training grants.

The fiscal year 1972 budget for research training will provide support in negotiated amounts, both for continuing and for renewal programs.

RESEARCH CAREER PROGRAM

Research Career Development Awards are made to nonfederal public or private institutions to enhance the career development of young scientists with outstanding research potential or demonstrated capability for careers of independent research in the sciences important to the cancer problem. In June 1964, the National Institutes of Health discontinued Research Career Awards which were made to enable institutions to finance positions favorable to the intellectual growth and research productivity of established investigators of high competence. Existing awards are being funded.

Effective May 1, 1969, it was permitted to supplement Research Career Development Awards by grantee institutions from nonfederal funds in an amount compatible with the institutional salary scale. In all cases the total salary to be provided an awardee must be justified according to the guidelines for salary support, as stated in the policy brochures and as hereby amended.

The maximum NIH contribution to the salary support will remain $25,000 per year. Fringe benefits and indirect costs, where permissible, may be requested only on the NIH contribution. All other terms and conditions of the awards remain in effect.

If a grantee institution desires to contribute to the salary support of any of its Career or Development awardees, the following information must be included:

1. The amount of the proposed supplementation.
2. A certification that the supplementation is from nonfederal funds.
3. A justification of the total salary, not just the NIH contribution.

There has been established a third deadline for the submission of new and renewal Research Career Development Award applications - December 1. Under this deadline, applications will normally receive notification of action by the following July.

The review schedule for RCDA applications will therefore be:

Deadline	Notification of Action By
December 1	July
May 1	December
September 1	April

A policy change dated February 5, 1971, stated:

1. A continuing review of the NIH Research Career Development Award Program indicates that a limitation of five years' duration on these awards will cover the critical period of the career development of each supported investigator.

2. Therefore, the present practice of renewing these awards will be discontinued. For those individuals now participating in the program, renewals will not be made if the expiration date occurs on or after June 30, 1972.

During fiscal year 1972, 11 Research Career Awards have been funded at a cost of $331,351 and 74 Research Career Development Awards at a cost of $1,742,617.

For the first time since the beginning of fiscal year 1970, we have been able to fund new development awards. Three new awards were made at the end of fiscal year 1971, and 14 new awards and two competing renewals have been made in fiscal year 1972.

There have been 12 terminations of development awards during fiscal year 1972:

10 came to the end of the award
2 changed institutions

The bulk of the candidates were, as formerly, nominated from medical schools.

During fiscal year 1972, the percentage of candidates not recommended for approval was approximately 23 percent with a slightly higher percentage of Ph.D.'s than M.D.'s.

Statistical analysis of geographic distribution shows that awardees are working in 22 states and the District of Columbia with the largest number in Pennsylvania:

Pennsylvania	12	Oregon	3
Massachusetts	10	Florida	2
New York	6	Kansas	2
Texas	6	New Jersey	2
Washington	6	Arizona	1
Wisconsin	6	District of Columbia	1
California	4	Nebraska	1
Connecticut	4	New Hampshire	1
Illinois	3	Oklahoma	1
Maryland	3	Tennessee	1
North Carolina	3	Utah	1
		Vermont 1	

Awardees of the National Cancer Institute are currently involved in 15 primary scientific disciplines. The largest numbers are conducting investigations in biochemistry and virology, with immunology, chemotherapy and morphology-morphogenesis following closely. Other disciplines in which the awardees are involved include enzymology, radiology, endocrinology, genetics, physiology, epidemiology, chemistry, cytogenetics and histology-cytology.

The number of applications for development awards assigned to the NCI has increased during the past year by approximately 50 percent. The calibre of the candidates has remained high.

Follow-up of individuals whose awards have terminated continues to show that the majority are still involved in research in the field of cancer. All of these former awardees have expressed the opinion that the award was very beneficial to them during tenure.

Efforts will continue to be made to encourage more qualified young investigators to move into the areas of radiation therapy and cancer epidemiology which are shortage areas in this program. Efforts will also be made to make the program known to small, less well-known institutions, encouraging them to nominate candidates for development awards.

FELLOWSHIPS PROGRAM

The fellowships program provides support to qualified American citizens for research training, to be obtained in this country or abroad, in the basic and clinical sciences associated with the cancer problem for the purpose of raising the level of competence and increasing the number of trained investigators and/or academicians in those biomedical sciences essential to the advancement of cancer research.

Postdoctoral support is offered to applicants who have earned a Ph.D., M.D., D.D.S., D.V.M., O.D., or equivalent degree. Special fellowships are offered to applicants who (a) have three or more years of relevant postdoctoral research experience, including residency or medical specialty training; (b) do not hold a doctoral degree but have demonstrated to the National Cancer Institute sufficient competence in their field to pursue their proposed training; or (c) who require special training, not provided by other programs, to meet particular program needs of the National Cancer Institute.

The following sub-paragraphs reflect the character of the 1971 program:

ACADEMIC LEVEL

Degrees held by fellows

Ph.D. - 157 D.V.M. - 8 (7 of these seek Ph.D.'s)
M.D. - 26 (one of these seeks Ph.D.)

Fellows according to number of years past receipt of doctoral at time of application

	1st yr.	2nd yr.	3rd yr.	4th yr.	5th yr.	6th yr.	7th or more yr.		Total Fellows
No. of Fellows	67	55	24	5	8	7	25	=	191

(The large number seven or more years past receipt of the doctoral, includes the M.D.'s who have completed residency training, and the senior investigators who are seeking special training during sabbatical leaves.)

GEOGRAPHIC DISTRIBUTION

Training in the United States - 181 fellows, training in 70 different institutions, in 27 states as follows:

54%

22% in California for about 20% of the total funds
12% in Massachusetts for about 13% of the total funds
10% in Maryland for about 13% of the total funds
10% in New York for about 9% of the total funds

Training in Foreign Countries - 10 fellows, training in 9 different institutions, in 4 foreign countries as follows:

	2% in England for about 3% of the total funds
3%	1% in Israel for about 2% of the total funds

(The remaining 43 percent of the fellows trained were too widely distributed geographically to obtain significant percentages.)

DISTRIBUTION BY DISCIPLINE

The 191 awards represent training in 21 different disciplines, which fall into the broad program areas shown below:

Cancer Biology - 84	Cancer Carcinogenesis - 45
Chemotherapy - 1	Epidemiology - 1
Immunology - 20	Pharmacology - 30
Radiology - 10	

The Institute recognizes that experience gained through training in a foreign laboratory with an eminent scientist is extremely valuable. For this reason, fellowship requests for such training have had priority in the assignment of the limited funds allocated to the Institute under the current foreign ceiling. Most of the new awards for foreign training during the past year were in the field of radiology and radiation therapy. In fiscal year 1971, one fellow was trained in England in fast neutron radiotherapy, an area with not only a shortage of trained personnel but also with few institutions which can provide this training. It is considered fortunate that two applications for this training are pending review in fiscal year 1972.

Program interests continue to encourage training in radiological and clinical radiation therapy as well as in the extensive and significant areas of chemistry.

Since fiscal year 1969, the financial condition has been precarious and complex as indicated by the following:

Fiscal Year 1969

Funds Available - $2,142,558 (includes supplements)
Awards - 234 for $2,142,558
Unfunded Approvals - 20 for $200,000

Fiscal Year 1970

Funds Available - $1,690,000 (includes supplements)
Awards - 180 for $1,690,000
Unfunded Approvals - 179 for $1,640,718 (only 3 with priorities beyond the 300 range)

Fiscal Year 1971

Funds Available - $1,797,000 (includes supplements)
Awards - 208 for $1,785,000
Unfunded Approvals - 54 for $500,000 (only 2 with priorities beyond the 300 range)

Fiscal Year 1972

Funds Available - $1,597,000 minus commitments - $1,151,023
Requested to Date - 164 for $1,445,683
Approved for Funding - 155 for $1,387,312
Awarded - 100 for $842,000
Unfunded Approvals - 55 for $545,312 (only 3 with priorities beyond the 300 range)

There are 108 applications still pending review for fiscal year 1972. Obviously, funding will be inadequate.

When the fiscal year 1970 budget was released, it was evident that in order to keep the program viable, future year commitments would have to be decreased. Consequently, during that year, when unfunded approvals almost equalled funded approvals in number and amount, only about 10 percent of the future years recommended were awarded.

In fiscal year 1971, support for two years was approved for 77 fellows. Only 26 of these were awarded second year support. The remaining 62 must reapply. These 62 represent about $504,000.

Thus far in fiscal year 1972, two year support has been approved for 80 applicants; 24 will be given a future year. Fifty-six must reapply.

Of the 108 pending applications, about 90 will be approved for two year support. About 26 will be awarded for two years. At the end of fiscal year 1972, there will be approximately 120 fellows approved for two years of support, but only one will have been awarded. This represents a decrease in the program in one year of 120 fellows for approximately $984,000.

The low budget has weakened the fellowships program disastrously, but more distressing is the deterioration of the program because of the poor stipends offered. The current National Institutes of Health stipend scale was established in 1967 and is among the lowest offered. Thirty-two of our fellowship awards were cancelled because the fellows received better support elsewhere. Six accepted American Cancer Society support; seven, Damon Runyon Foundation support; and seven accepted other foundation support: Helen Hay Whitney, Jane Coffin Childs, American Leukemia, et al. Seven others accepted positions and the remaining five accepted unidentified support.

The average priority of these 32 cancellations was 171, the funds $219,000.

With fiscal year 1972 not yet ended, we have 31 cancellations with an average priority of 165. We may expect a dozen more, all in the 100 priority range.

In fiscal year 1971 cancellations were 12 percent of the total approvals. So far in fiscal year 1972 cancellations have already risen to 20 percent of approvals.

Funds freed by the cancellation of our top priority awards are used to make awards which were originally unfunded. The more cancellations, the further down the priority list funding goes, decreasing the number of unfunded approvals. The resulting contradiction is that a reduction in the budget seems to have brought a reduction in unfunded approvals.

End-of-the-year statements do not reveal these program weaknesses. The one-year awards represent a 120-fellow-a-year loss to the program in addition to the loss of 20 percent of the top priority awards through cancellations.

The remedy is an increase in fellowship stipends and in the fellowship budget. A stipend increase of $2,000 for each postdoctoral level is desirable whether or not there is a much needed budget increase in fiscal year 1973.

CANCER CORE ENRICHMENT PROGRAM

Guidelines have been developed for a new type of grant program to be known as the Cancer Core Enrichment Program. These grants would be awarded to schools of medicine, dentistry and public health, specialized cancer institutions, major teaching hospitals, and selected non-profit, non-federal health or professional organizations for the purpose of:

(1) Determining defects within existing patterns or systems of health care with regard to early detection and/or diagnosis of cancer;

(2) Determining what remedial steps may be initiated to correct and repair these defects;

(3) Effecting within a given area a demonstrable reduction in cancer morbidity and mortality.

Support would be offered through the grant mechanism to institutions and organizations whose administration and staff members demonstrate broad insight into the cancer problem and who have access to sufficient personnel and resources so as to plan and conduct such projects effectively. It is not the purpose of these awards to support ongoing activities within an institution, but additional activities which will fulfill the above goals.

Cancer Core Enrichment grants would be awarded only when favorably recommended to the National Cancer Institute by the National Cancer Advisory Board. Technical advice on all such grants would be provided to the Board by a Cancer Core Enrichment Committee, whose members would be selected because of their knowledge and competence concerning the clinical aspects of cancer, as well as the organization and administration of health service studies.

Cancer Core Enrichment projects should represent a coordinated effort within the applicant institution (organization) to study and improve the pattern of referral among the individuals within its purview who have cancer. Existing patterns of referral may be used as baselines from which to plan measures which would reduce morbidity and mortality due to one or more types of cancer in the referral area or community.

The initial studies should not be limited to the data in the primary institution (organization) but should include that of others within the referral area. Likewise, the proposed remedial activities should be directed toward the area served and not limited to the primary institution.

Administrative:

An outline of the Cancer Core Enrichment Program was presented to the National Advisory Cancer Council at its meeting in November 1971, and was endorsed by that body, which recommended that the Program be implemented as soon as feasible. In the National Cancer Institute's budget for fiscal year 1973, funds were requested to launch this new Program. This request was however, subsequently deleted from the President's budget.

Guidelines for the new Program have been developed, and a synopsis has been prepared for insertion in the Federal Register. When funds permit, the Program will be initiated.

The cancer research Centers Program was activated approximately a decade ago. As a result there is increasing cancer research, training, and education being carried on in the medical schools, rather than being confined largely to specialized cancer hospitals and research laboratories. The complexities of the various neoplastic diseases and the need for an increased understanding of the fundamental processes involved require a coordinated effort on the part of scientists ranging from those undertaking basic research in cancer biology to research clinicians, and finally to clinicians responsible for the care and treatment of cancer patients. In order to achieve this, the medical schools, specialized cancer facilities, practicing physicians, and community medical services must participate in an integrated multi-disciplinary program. This will enhance the extension of the most advanced diagnostic and therapeutic techniques to all cancer sufferers.

With experience, these multidisciplinary research programs have become more complex and varied in stages of development, and three types of center activities can now be recognized. Briefly, the simplest is the intradepartmental program which includes radiation therapy centers, in which several disciplines work together within an administrative unit of an institution. The research is usually targeted toward a specific problem area and closely coordinated. The next, more complex, is the interdepartmental program, in which several semi-autonomous units within an institution or school combine efforts to work on problems of broader scope. Clinical research frequently requires such interdepartmental cooperation. Comprehensive, institution-wide programs are the most complex, and these are evolving to include more and more involvement with community institutions and health practitioners, thereby encouraging the more rapid application of research findings. These comprehensive cancer centers are considered to be the most efficient arrangement for sharing costly resources and for enhancing cancer capability.

Since there is a fine line that distinguishes one type of center from another in most instances, it is not easy to make a comparison among them. Also, since they are for the most part functional rather than physical or organizational units, it is not always clear as to what degree certain activities are an integral part of the center as opposed to other activities within the institution. A solution to the above might be to have a more centrally directed centers program. However, this could result in the loss of flexibility which allows for changes in centers programs and activities as new developments occur in cancer research and therapy.

The research centers provide a unique training ground for physicians, paramedical personnel, and students. Many of them are involved in the cooperative clinical trials of the NCI Clinical Investigations Branch. Others utilize their own therapy protocols. For these reasons, and many

others, the need to create new centers and strengthen or expand existing ones continues. In this regard, geographic distribution has been an important consideration. New centers have been established in the South and West in an effort to provide more uniform resources to communities throughout the United States. The National Cancer Institute in fiscal year 1972 is supporting 37 clinical center grants for approximately $21 million, of which 12 are Radiation Therapy Centers. Included in the above are six new cancer clinical center grants, four more than in fiscal year 1971, totaling an estimated $4.3 million. Forty-two program center grants, which are primarily concerned with basic research, will be funded in fiscal year 1972, totaling approximately $25.5 million. There are six approved but unfunded program center grants as fiscal year 1972 ends.

An element that has served to focus attention and interest on cancer centers has been the deliberations leading to the passage of the National Cancer Act of 1971 in December of that year. The Act provides not only for the strengthening and expansion of existing centers, but also contains a specific section which authorizes the establishment of "fifteen new centers for clinical research, training, and demonstration of advanced diagnostic and treatment methods relating to cancer." This section provides that payments may support construction, staffing and other basic operating costs, including such patient care costs as are required for research and training, including training for allied health professions personnel, and demonstration purposes. The emphasis on demonstrating improved patient care has encouraged many community institutions providing care to cancer patients, in addition to medical schools and health centers, to become interested in active participation or sponsorship of cancer centers. This was further reinforced through the jointly sponsored (NCI and the American Cancer Society) meeting December 9 and 10, 1971, on planning cancer centers.

In order for an institution to establish, develop, and operate the above kinds of cancer centers, extensive planning is required. It is important that the planning effort carefully examines and analyzes the various alternatives which exist for the institution to incorporate the center program into its organizational structure. The entire effort should assist in selecting the most desirable course of action for the institution to accomplish its goal, a well-defined, organized and effective cancer program. This type of planning is not only difficult and time consuming, but also rather expensive. Therefore, the National Cancer Institute participates in planning costs through exploratory grants. In fiscal year 1972, 19 exploratory grants are being supported, eight of which were initiated this year. Many of these awards are to institutions located in geographical areas where centers do not now exist. The total amount for support of these grants will be approximately $1.9 million. There are 24 approved, unfunded exploratory grants as of April 1, 1972.

CANCER RESEARCH AND TRAINING FACILITIES CONSTRUCTION PROGRAM

The Cancer Research and Training Facilities Construction Program was initiated in late September 1971 with a public announcement of the availability of limited grant funds to create new cancer research facilities. The essence of this announcement is set forth in the following paragraph:

> In accordance with the President's call for an expanded, intensified, and coordinated cancer research program, and under authority provided by the Congress in the fiscal year 1972 appropriation act, the National Cancer Institute is initiating a program of grant supported construction of cancer research facilities. The intent of this program is to create new physical resources for cancer research through Federal participation in the cost of new construction and of renovation. Support may be provided for the construction of facilities such as basic research laboratories; clinical research facilities; animal facilities; and associated core, administrative, laboratory, and service space. In all instances, the facilities proposed must be intended for expansion of cancer research and must be used for cancer research for at least 20 years. A purpose of this program in addition to strengthening research capabilities at existing cancer centers is to develop new strong multidisciplinary cancer efforts in regions of the country where they do not now exist. Thus, in the consideration of applications some attention will be given to geographical distribution as well as to the relation of proposed construction to existing centers of excellent cancer research.

Legislative authority was later established in the National Cancer Act of 1971, Sec. 410(2).

November 1, 1971 was set as the deadline for the acceptance of construction grant applications, and by shortly after that date 22 applications had been received for review. Of these, 20 applications were subsequently reviewed (including site visits) and presented to the National Cancer Advisory Board for consideration at the March 1972 meeting. One of the 22 applications was withdrawn; the other was administratively deferred at the request of the applicant.

Since justification for construction funds is in terms of new cancer research programs, the primary review and evaluation of the applications was essentially scientific, considering such matters as scientific merit of the program(s) proposed, the technical competence of the applicant institution staff, the intellectual environment of the applicant institution, and the scientific and fiscal administrative capabilities of the applicant institution. In addition, other criteria were considered, such as the geographical location of the applicant institution, applicant institutions performing an essential role in the National Cancer Program, the promptness with which construction can be gotten underway, space request commensurate with projected program scope, essential minimal facilities for those institutions

with an undeveloped cancer research program, space utilization over 60 per cent, etc.

Subsequently, the review and evaluation of these initial applications was accomplished by 20 ad hoc Special Review Committees (involving 88 scientists) between January 1 and March 1, 1972. On March 17, 1972, the Chairmen of these Special Review Committees were convened as a Committee to reconsider all reviews and establish an order of priority, which in their judgment was most applicable and consistent with the National Cancer Program. The recommendations of the Special Review Committees and the Committee of Chairmen were forwarded to the National Cancer Advisory Board for its consideration on March 20-21, 1972.

The results of the reviews by the Special Review Committee are of interest. Of the 20 applications, 14 were recommended (one on a split vote) for funding, for a total of $35,643,988. One application was deferred, pending a decision by the National Cancer Institute regarding the role the applicant institution can play in the National Cancer Program; five applications were recommended for disapproval on the basis of inadequate research program justification. The Committee of Chairmen, after reconsidering all reviews, recommended approval of 13 applications, deferral of two, and disapproval of five (the same five recommended by the Special Review Committee).

The National Cancer Advisory Board concurred with all but two of the recommendations of the Committee of Chairmen. Decisions were made to (a) recommend funding one of the deferrals (as originally recommended by the Special Review Committee), and (b) defer one of the disapproved applications pending a Board site visit prior to the June 1972 meeting.

The final recommended total funding level for the approved applications is $34,564,856. The original requested funding level for the 20 applications was $64,286,932. The order of priority recommended by the Committee of Chairmen, and concurred with by the Board, has become extremely important for staff guidance in allocating the $11.0 million in construction funds currently available. However, the Board was advised that adequate supplemental construction funds may become available so that all approved applications can be funded.

February 1, 1972 was the next deadline for new construction grant applications. Seven applications were received for a total requested funding level of $25,747,366. These applications will be reviewed by ad hoc Special Review Committees. Their recommendations will go forward to the National Cancer Advisory Board for the June 1972 meeting.

The acquisition of staff to accomodate this new program has been slowly progressing. Adequate professional staff is available, as is supporting staff. Office space is critical at the present time and will become even more so as new staff is added.

Authority has been given the National Cancer Institute to establish a permanent Cancer Research and Training Facilities Construction Review Committee.

The necessary actions to form this Commitee are now underway, and it is estimated the Committee can be activated July 1, 1972.

Program projections into fiscal year 1973 are difficult to make at this time. On the basis of what has been received thus far, it is likely that as many as 50 construction grant applications will be received for review and evaluation during the next fiscal year. Using an average request funding level of $3.3 million (i.e., based on the applications received so far), we can expect a total requested funding level of approximately $150.0 million. Projecting this further, using the experience of our limited review recommendations, the recommended funding level will probably be reduced to approximately $78.0 million. It must be emphasized that these projections are only approximations at best.

NATIONAL ORGAN SITE CANCER PROGRAM

The National Organ Site Cancer Program consists of grant-supported national projects of targeted cancer research. Each national project is a planned and integrated research effort oriented toward cancer at a specific organ site. The planning, direction, coordination, and scientific administration of a national project is conducted at a headquarters institution other than the National Cancer Institute. The Program Director, who is not an employee of the National Cancer Institute, is assisted in the planning and in the scientific administration of the national project by a Working Cadre of scientists recruited from institutions throughout the Nation.

The purposes of this program are: (1) to prosecute grant-supported national projects of planned and coordinated research in areas that are ripe for exploration, (2) to capitalize on the scientific talent and management expertise in institutions throughout the Nation, and (3) to decentralize the day-to-day scientific management of grant-supported programs.

Each national organ site project is initiated with a planning phase in which a Program Director and a Working Cadre develop a comprehensive and detailed long-range plan of research directed toward improving prevention, diagnosis, and treatment of cancers of a particular organ. When this comprehensive research plan is prepared, an advisory committee reviews the plan and makes recommendations to the National Cancer Advisory Board. If the Board favors implementing the plan, it recommends an overall amount which in its view is adequate for funding projects within the plan. Upon receiving this recommendation, the National Cancer Institute determines the amount of support to be allocated to the Working Cadre for the national project.

In the operational phase of a national organ site project, a Program Director with the aid of his Working Cadre solicits research proposals, coordinates the review of applications, and based on the review, recommends to the National Cancer Institute which projects should be funded. The National Cancer Institute acts upon this recommendation without further review of individual applications. However, an advisory committee provides a continuing assessment of both the scientific quality and the administrative excellence of the national project.

Three Working Cadres began planning during fiscal year 1972: (1) the Bladder Cancer Working Cadre under the direction of Dr. Gilbert H. Friedell, St. Vincent Hospital, Worcester, Massachusetts; (2) the Large Bowel Cancer Working Cadre under the direction of Drs. Murray M. Copeland and Walter J. Burdette, M.D. Anderson Hospital and Tumor Institute, Houston, Texas; and (3) the Prostatic Cancer Working Cadre under the direction of Dr. Gerald P. Murphy, Roswell Park Memorial Institute, Buffalo, New York. The latter group was organized late in the fiscal year but succeeded in initiating its planning activities.

Two advisory committees were established to review and evaluate the activities of the Working Cadres: (1) the Bladder-Prostate Cancer Advisory Committee under the chairmanship of Dr. Rubin H. Flocks, University of Iowa; and (2) the Colon-Rectum Cancer Advisory Committee under the chairmanship of Dr. Joseph F. Ross, University of California, Los Angeles. Both of these committees have been active in reviewing plans developed thus far. The National Cancer Advisory Board in its first meeting, March 1972, concurred with the recommendation of the Bladder-Prostate Cancer Advisory Committee that implementation of the National Bladder Cancer Project proceed.

The plan which the National Bladder Cancer Project began implementing in the latter part of the fiscal year encompasses the general areas of epidemiology, experimental biology, diagnosis and prognosis, prevention, detection, and treatment. In epidemiology, the major areas of interest are in the identification of populations with differing risks of bladder cancer, the identification of factors associated with differing risks, and identification of etiological factors in bladder cancer through analytical studies. In experimental biology, the plan calls for the improvement, development, and exploitation of experimental models for bladder cancer. The general areas of concern are the identification and/or development of *in vivo* and *in vitro* experimental models for studies of induction, biology, and therapy of bladder cancer; the investigation and characterization of the specific biologic attributes of the experimental models; and the utilization of these experimental models to study prevention, detection, and treatment. The major areas of interest in diagnosis and prognosis are in formulating and improving existing morphological criteria for diagnosis, the development of non-morphologic criteria for diagnosis, and in the development of a system of classifying bladder cancer host interaction for prognosis. In prevention, the plan intends to draw upon information to be developed from the epidemiological studies and from the study of experimental models. This information will be used to develop measures to prevent or inhibit the initiation or growth of bladder cancer, and once having developed such measures, to determine their effectiveness by controlled intervention studies as well as by population studies. In the area of detection, the broad objectives are to develop and test cytologic, biochemical, immunologic, and other detection techniques. The initial emphasis will be on improving the current techniques of exfoliative cytology and attempting to make the technique practical for large-scale utilization. The general objectives in treatment are to evaluate, improve, or develop chemical, immunologic, radiologic, and surgical techniques.

CANCER SPECIAL PROGRAM

The Cancer Special Program Advisory Committee (CSPAC) has maintained continuous review and monitoring of the single-support grant (R01CA 08748-06) to the Sloan-Kettering Institute (SKI) during the past year. The Committee was particularly concerned with the abilities of the interim administration of SKI during a period when the institute was without a director; hence, without specific scientific leadership.

The estimates of the Committee, relative to the probable effectiveness of an "acting" administration were thoroughly discussed in the 5th YEAR REPORT completed and forwarded to the NCI Director in February 1971. It was the opinion of the Committee at that time, that in a period of emergency, the "acting" administration was capable of maintaining the status quo, but probably would not effect too many changes in the scientific direction of the total programs and/or projects within the total program.

The Committee met in mid-July 1971 to review and evaluate a renewal application for the 08 year (i.e., calender year 1973). That review confirmed the initial judgment of the Committee relative to the capabilities of the "acting" administration, for it had been in effect approximately five months since the untimely death of Dr. Frank Horsfall the previous February. The "acting" administration was holding the activities together in an effective manner, and would probably be able to do so for some time to come, but very little influence was being exerted on the scientific direction of the total program and/or its contents, except within circumscribed limits by the Vice Presidents and Division Chiefs.

The Committee, after considering other aspects of the SKI program, approved the application with certain stipulations, and forwarded its recommendations to the National Advisory Cancer Council (NACC) in November 1971. The Council concurred with the essential recommendation of approval, but decided that funds for new programs should be requested by supplemental applications and reviewed by CSPAC.

As a result of this action, and an earlier action by NACC at the June 1971 meeting relative to the funding level for the 07 year (approved by NACC in November 1969), SKI now has committed support until December 31, 1973. Presumably during this period the new SKI Director, Dr. Robert Good, will consider the single-support mechanism, along with alternative funding procedures and will decide how he will like to have SKI programs supported in the future.

In the late fall, Dr. Chester S. Keefer resigned as chairman of the Committee because of health reasons (Dr. Keefer died in February 1972) and he was succeeded by Dr. Charles W. Todd.

The Committee intends to continue monitoring the current grant during the fiscal year 1973, and will stand ready to assist SKI and NCI in any rearrangements which may be made.

APPENDIX I

Published Results of Scientific Work

Supported by the Extramural Programs

of the

National Cancer Institute

July 1, 1971 through June 30, 1972

PUBLISHED RESULTS OF SCIENTIFIC WORK SUPPORTED BY THE EXTRAMURAL PROGRAMS OF THE NATIONAL CANCER INSTITUTE

The compilation of scientific data and the interrelating and interpretation of information to create valid knowledge, is a difficult task at best. In a diverse field such as cancer research, which encompasses all disciplines of medicine, this task is extremely complicated. However, because of the very complex nature of cancer, it is necessary from time to time to attempt to relate and interpret the diverse information and assess the knowledge gained concerning the disease.

During the fiscal year 1972 approximately 1800 NCI extramural awards have been made. This report, prepared by the Program Analysis and Reporting Section, OADEA, presents a correlation of selected portions of research supported by those awards and which have been published during the last year. The source of individual NCI extramural support for each paper cited appears as a notation with each reference in the form of the award number or numbers; also symbols of the appropriate extramural program areas are included.

CHEMICAL CARCINOGENESIS

Years of systematic analysis of known chemical carcinogens and related compounds have yielded a detailed pattern of characteristics associated with carcinogenicity. Fuller delineation of the physico-chemical properties which determine carcinogenic activity should help to reveal the mechanisms of action of chemical carcinogens and, possibly, suggest ways to block or reverse such activity.

Carcinogenicity of N-acyl-2-fluorenylhydroxylamines in the rat appears to depend on prior metabolic deacylation of these compounds to the N-2-fluorenylhydroxylamine. In addition to the size of the aryl moiety and the position of the acyl group relative to the aromatic system, carcinogenicity of N-acyl-2-fluorenylhydroxylamines apparently depends on the ease of removal of the acyl or benzenesulfonyl group from the molecule. Replacement of the hydrolyzable acyl or benzenesulfonyl group by a phenyl group results in a molecule with greatly diminished carcinogenic properties.[1] Administration of N-hydroxy-2-fluorenylbenzenesulfonamide (N-HO-2-FBS) to rats yielded several urinary metabolites that are very likely derived from N-2-fluorenylhydroxylamine.[1] There appeared to be a correlation between the extent of desulfonylation of N-HO-2-FBS and its site of action, since the reaction proceeded at a faster rate in the mammary gland, which is the primary target of N-HO-2-FBS, than in the liver or the kidney of the female rat. N-phenyl-4-biphenylylhydroxylamine was not carcinogenic either for the male or female rat. These as well as previously published data support the view that N-2-fluorenylhydroxylamine is an obligatory intermediate for the carcinogenicity of N-acyl-2-fluorenylhydroxylamines.

3-Methylcholanthrene (MC) inhibits the hepatocarcinogenic action of N-2-fluorenylacetamide (AAF) but not of N-hydroxy-AAF (N-HO-AAF) in the rat. Low levels of MC caused a 45% decrease in binding of radioactive label to DNA and rRNA after injection of AAF-9-^{14}C into rats, but no decrease after injection of N-HO-AAF-9-^{14}C.[2] These results were consistent with the suggestion that MC inhibits AAF carcinogenicity by decreasing intracellular levels of N-HO-AAF by

preferential induction of ring hydroxylase relative to N-hydroxylase activity in the hepatic endoplasmic reticulum. However, MC apparently also exerts some influence on a later reaction involved in the metabolism of N-HO-AAF, but this effect is seen only with a large single injection of MC.[2] This later action is apparently responsible for inhibition of N-HO-AAF binding to liver nucleic acids and proteins.

N-HO-2-AAF is esterified in rat liver to a sulfate which reacts with nucleophiles such as tRNA, methionine, and methionyl residues in peptides and proteins. N-HO-3-AAF, an isomer of N-HO-2-AAF, is a potent mammary carcinogen for both female and male rats when administered intraperitoneally. A recent report[3] indicates that N-HO-3-AAF is apparently either not esterified to a sulfate or is esterified to a sulfate which is unreactive toward tissue nucleophiles. A mechanism of action for mammary carcinogenesis involving esterification of N-HO-3-AAF would require a sulfotransferase with a substrate specificity different from that of liver or a different type of ester. The data raise the question of whether the same molecular mechanism that applies to the induction of neoplasia by arylhydroxamic acids in hepatic tissue accounts for the carcinogenicity of these compounds in extrahepatic targets. Because of the reactivity and instability of AAF-N-sulfate, which appears to be one of the ultimate products of 2-AAF and N-HO-2-AAF in rat liver, it is extremely unlikely that this metabolite is transported from the liver to extrahepatic tissues. Therefore, extrahepatic carcinogenesis by 2-AAF and its metabolites, as well as by N-HO-3-AAF, may well occur by some other mechanism than that which is active in liver. Experimental evidence supports this view. AAF-N-sulfate was not formed[4] in vitro in the mammary gland or in the sebaceous gland of the external auditory canal (Zymbal's gland), tissues of the rat that are highly susceptible to carcinogenesis by 2-AAF and N-HO-2-AAF. The inability of these tissues to form AAF-N-sulfate appears to be due to lack of a sulfotransferase which can use N-HO-AAF as a substrate, rather than to the presence of an inhibitor of the reaction. On the basis of current knowledge of the metabolism of AAF and N-HO-AAF, several candidates for the proximate metabolite of these carcinogens are suggested:[4] (a) N-HO-AAF itself, which has been reported to have limited but detectable reactivity with nucleic acids in vitro; (b) the glucuronide of N-HO-AAF or of N-HO-AF; and (c) N-HO-AF.

Preliminary testing has shown the O-glucuronide (N-GlO-AAF) of N-HO-AAF to be only weakly carcinogenic. However, results obtained[5] by testing N-GlO-AAF in both tricaprylin and 0.9% NaCl show that the choice of solvent may greatly influence the outcome of a carcinogenesis assay, particularly when the test compound is administered by subcutaneous injection. No tumors were produced in rats in which tricaprylin was used as a vehicle for administration of N-GlO-AAF as a suspension. However, when injected s.c. as a solution in 0.9% NaCl, N-GlO-AAF induced a total of 9 tumors in 16 rats: one sarcoma at the site of injection and eight tumors at distant sites. These results definitely establish the carcinogenicity of N-GlO-AAF for the rat.

Administration of chemical carcinogens to various mammalian species causes covalent binding of these parent compounds or their metabolites to susceptible tissue macromolecules. There is a direct relationship between the degree of carcinogenicity of these chemicals and their macromolecular binding but there

is no definitive evidence that would indicate that any particular macromolecular binding is involved in the initiation of cancer. In view of the suggestion that non-histone nuclear proteins such as acidic proteins may play a role in the regulation of gene expression and function in mammalian cells, an attempt was made[6] to determine whether these acidic proteins are involved in AAF binding in vivo. When rat liver nuclear proteins were fractionated, the specific radioactivity of the sulfuric acid-insoluble fraction (which would be composed mainly of acidic proteins) was found to be about four times that of the sulfuric acid-soluble fraction. The fluorene residues were bound to nuclear acidic proteins to a much greater degree than to histones. In view of these results and the suggestion that these proteins may regulate gene expression, it appears that modification of these acidic proteins by arylation with fluorene residues might alter their regulatory properties. It is possible that such modifications of acidic proteins may play a role in the initiation of liver cancer by aromatic amines.

The conformational properties of various oligonucleotides modified by AAF have been investigated[7] by utilizing circular dichroism, proton magnetic resonance spectroscopy, and computer-generated molecular models. Introduction of the carcinogen, specifically and covalently, at the C-8 position of guanosine residues results in dramatic changes in the circular dichroism spectra of the oligonucleotides. The attachment of AAF also causes large higher-field shifts for the proton resonances of fluorene and bases adjacent to a modified guanosine residue. These results, together with substantial supporting evidence, show that the covalent binding of AAF causes important changes in the conformational properties of oligonucleotides in aqueous solution. The major changes include rotation of the guanine base around the glycosidic linkage and the intramolecular stacking of fluorene with an adjacent base. The specific conformational changes noted for the oligonucleotides could clearly disrupt the normal biological activity of similarly modified naturally occurring nucleic acids.

Because of the central role of the genome in regulating cellular processes, and the large number of agents which react with DNA, it would appear that an understanding of DNA repair processes in chemical and enzymatic terms is important in the metabolic and cellular analysis of carcinogenesis. Thymidine incorporation into human peripheral blood lymphocytes was studied[8] after exposure of the cells to alkylating agents, precarcinogens, proximate carcinogens, and noncarcinogens. In the presence of hydroxyurea to suppress S-phase DNA synthesis, cells treated with nitrogen mustard, methyl methanesulfonate, ethyl methanesulfonate, or with the proximate carcinogens β-propiolactone or N-acetoxy-2-AAF, incorporated 4-9 times as much thymidine as did controls. No stimulation was noted with the precarcinogens dimethylnitrosamine, 3'methyl-4-dimethylaminoazobenzene, and AAF, or with iodoacetamide which alkylates protein but not DNA. HeLa cells infected with Sendai virus appear to have an enhanced capacity for repairing both their own and herpes simplex viral DNA damaged by the potent carcinogen 4-nitroquinoline 1-oxide (4NQO).[9] This result is interpreted as suggesting that the observed differences in repair activity are due to repair enzymes other than those involved in excision of damaged DNA segments. In fact DNA polymerase I activity of Sendai virus-infected cells was twice as high as

in uninfected cells. Bacterial DNA polymerase I has been reported to be involved in repair of phage genomes damaged by 4-hydroxyaminoquinoline-1-oxide (4HAQO), a reduction product of 4NQO.

4NQO inactivates bacteria, but not bacteriophage, while 4HAQO inactivates both, suggesting that the latter compound interacts directly with DNA. A method for detecting the activating enzymes that convert 4NQO to 4HAQO or a reactive intermediate that inactivates phage in the presence of enzyme fractions, NADH, and 4NQO has recently been described.[10] Only one protein peak from Salmonella typhimurium was able to cause phage inactivation; this same peak was the only one with bacterial diaphorase and 4NQO-reducing activity. Similarly, experiments with rat liver cytosol fractions showed that there are two peaks that activate 4NQO, and these also corresponded to diaphorase activity. These results suggest that diaphorase is an activating enzyme for 4NQO.

The concept that the carcinogenic 4HAQO's interact with DNA by a mechanism other than covalent bond formation can be supported by several lines of experimental evidence. Moreover, in view of the substantial agreement found[11] in a series of 4NQO's and their derivatives between known carcinogenicity and molecular orbital calculations interpreted with the use of a steric model, the 4NQO's themselves must be considered as potentially having a direct role in carcinogenesis. If the suggested model of the binding of 4NQO's and 4HAQO's to deoxyguanosine in DNA is biologically significant, these compounds may cause erroneous base selection during DNA replication. Such binding to DNA might also inhibit DNA-dependent RNA synthesis as suggested by the results of another study on the mechanism of action of 4NQO. 4NQO and several related carcinogenic and noncarcinogenic derivatives were tested[12] for their ability to produce nucleolar segregation in vitro in Chang liver cells. The results indicate that there may be a relationship between nucleolar segregation and carcinogenicity among the NQO's and that this effect may be mediated by inhibition of RNA polymerase.

Studies of hydrocarbon metabolism in cultured cells from several mammalian species suggest that there may be sequential conversion of the parent hydrocarbon first to alkali-extractable derivatives and then to water-soluble (aqueous-acetone-soluble) derivatives. A study of the relationship between carcinogenicity and cytotoxicity (growth inhibition) showed[13] that all the cell cultures tested that were sensitive to the growth inhibitory effects of 3,4-benzpyrene (BP) or 7,12-dimethylbenz(a)anthracene (DMBA) were able to metabolize these carcinogenic hydrocarbons to "water-soluble derivatives". In general, little or no ability to metabolize the hydrocarbons was detected in cells which were resistant to cytotoxic effects, although the hydrocarbons were readily taken up by these cells. The results are consistant with the hypothesis that metabolism of these carcinogens is required for their cytotoxic effects and that metabolism to cytotoxic derivatives is mediated, at least in part, by aryl hydrocarbon hydroxylase. The fact that 1,2-benzanthracene (BA) is metabolized as well as BP or DMBA, but is relatively non-cytotoxic, demonstrates that metabolic conversion alone is insufficient for cytotoxicity. The types of products formed and their ultimate fate more probably determine the cellular response. In terms of biologic activity the metabolites remaining within the cell may be more im-

portant than those which are excreted. This would be particularly true if binding of hydrocarbon metabolites to cellular macromolecules were required for the cytotoxic or carcinogenic activity of these compounds.

The cytotoxicity and mutagenicity of several polycyclic hydrocarbons and their K-region derivatives were tested[14] in clones of Chinese hamster cells; production of clones resistant to 8-azaguanine was used as the marker for mutagenesis. All the K-region epoxides tested were strongly mutagenic. All phenols were somewhat mutagenic and the one derived from dibenz(a,h)anthracene was more mutagenic than the corresponding epoxide. K-region epoxides have also been shown[15] to be more active in producing in vitro malignant transformation than the corresponding hydrocarbons, dihydrodiols, and phenols. Because of their chemical reactivity, until now epoxides had never been isolated as metabolic products of carcinogenic hydrocarbons. It has now been demonstrated[16] that an epoxide is produced in the microsomal metabolism of dibenz(a,h)anthracene (DBA) in rat liver. This finding strengthens the view that epoxides are the activated metabolic and the ultimately carcinogenic derivatives of carcinogenic hydrocarbons, at least of those that lack methyl groups.

The high susceptibility of the female rat mammary gland to chemical carcinogens promoted a study[17] of DMBA binding to DNA and protein in female rat mammary gland parenchymal cells. Fifty percent of the DMBA bound to DNA 16 hrs after administration was still present on the 14th day, and 31% was still detectable at 42 days. The amount of DMBA binding to protein was less than one-half that observed bound to DNA. Furthermore, by 14 days, DMBA binding to protein had declined to barely detectable levels. This persistent binding of DMBA to mammary parenchymal cell DNA in vivo may be responsible for the depression of RNA polymerase activity which has been demonstrated in vitro. A method was developed[18] for isolation of rat mammary gland cell nuclei and DNA-dependent RNA polymerase activity in isolated mammary gland nuclei was measured[18] in rats fed a single dose of DMBA. In female rats, there was a significant inhibition of Mn^{2+}-$(NH_4)SO_4$-activated RNA polymerase activity within 24 hr after the feeding of DMBA. Inhibition continued for 2 days, but thereafter the enzyme activity rose significantly above the control value. In contrast, RNA polymerase activity was not depressed in male rats treated with DMBA.

In another study of DMBA binding to cell macromolecules the in vivo binding of DMBA to the DNA, RNA, and protein of rat mammary gland, as well as the nature of these bindings, was investigated.[19] The amount of DMBA bound to the cytoplasmic soluble protein was remarkably high in comparison with other cellular constituents. DMBA was bound minimally to RNA fractions as well as to nuclear and ribosomal proteins. In contrast, the noncarcinogen anthracene was bound to a greater extent to DNA than to the other cellular constituents. The nature of the binding rather than the amount of hydrocarbon bound may be more relevant to its carcinogenicity. About 6% of DMBA bound to DNA occurred by hydrophobic or other noncovalent binding and 14% (20% when corrected for the 6% due to hydrophobic binding) was probably buried in the macromolecule matrix and became soluble after digestion with DNase. Apparently 80% of the DMBA which was bound was due to covalent binding; this fraction was resistant to benzene extraction either after DNase digestion or after heating to 80°. In contrast, about 80% of anthracene was bound to DNA by means of intercalation,

and the remaining was hydrophobic (12%) and covalent (8 %). There was no significant difference in binding to protein between DMBA and anthracene. About 80% of these bonds were covalent and resistant to benzene extraction even after digestion with pronase.

In recent years, increasing attention has been focused on the ability of 3-methylcholanthrene (MCA) to alter the activity of a group of closely related microsomal enzymes which function in the metabolism of drugs and other foreign compounds. The enzyme systems affected by MCA may also function in the metabolism of other hormonal substrates such as estrone, hydrocortisone, and testosterone, as well as thyroxine (T_4). Data presented in a recent report[20] are consistent with the hypothesis that the rate of hepatic conjugation of T_4 is accelerated in MCA-treated rats, which results in an increased rate of biliary secretion and an increased fecal excretion of T_4. Four macromolecules in rat liver cytosol bind corticosteroids and their anionic metabolites. One of these, a basic protein designated "corticosteroid Binder I" has been shown[21] to bind dimethylaminoazobenzene. It now appears that it binds MCA also.[22] Although the function of the protein is presently unknown, it could act in the metabolism of the carcinogens or in their transport to a site of metabolism.

The vast majority of work with the microsomal enzyme systems has been carried out with liver although current evidence indicates that they occur in the tissues of all major portals of entry including the gastrointestinal tract, lung, skin and placenta. Most, and possibly all, of the aryl hydrocarbon hydroxylase activity in the small intestine of the rat appears to be due to exogenous inducer in the diet.[23] Induction was obtained[23] with several members of the Brassicaceae family, including brussel sprouts, cabbage, turnips, broccoli, and cauliflower, and was also found with alfalfa and spinach. The implications of this study appear to be of some consequence with regard to the response to exposures to polycyclic hydrocarbon carcinogens. The amount of inducer(s) in the diet would be capable of altering the capacity of the organism to metabolize these compounds.

Dietary components may be important not only in the metabolism of chemical carcinogens, but also in their formation. The possibility that nitrosamines, many of which are powerful carcinogens, can be formed in the stomach of mammals from ingested amines and nitrites has led to speculation on the sources of relevant amines for man. One such source is the large number of drugs that are secondary or tertiary amines and many of which are administered on a long-term basis. Phenmetrazine (3-methyl-2-phenylmorpholine) was found[24] to be nitrosated by rabbits and rats to which it was administered with sodium nitrite. The carcinogenicity of nitrosophenmetrazine was not tested but because nitrosomorpholine is a potent liver carcinogen in rats, it would be unwise to assume that the derivative formed in this study is noncarcinogenic.

VIRAL CARCINOGENESIS

There is overwhelming, although only indirect, evidence that viruses are involved in the development of human cancer. This is not unexpected in view of the wide range of species in which viruses have been demonstrated to cause

cancer. Whether any of the viruses which have been shown to cause tumors in other species are able to do so in humans is unknown and the relationship between these viruses and those isolated from human tumors is under continuing investigation.

Disseminated rhabdomyosarcomas with human karyotype developed in four kittens following prenatal inoculation with a line (RD cells) of human rhabdomyosarcoma cells.[25] Although the parent cell line contained no detectable C-type virus particles, such particles were found in two of the induced tumors and in a cell line (RD-114) derived from one of these tumors.[26] Virus in filtered culture fluids from the RD-114 cell line failed to transform human, cat, dog, mouse, or rat embryo cells in preliminary experiments using a standard transformation assay method. The virus did replicate in human and dog cells. In repeated tests at several passage levels, sonicated RD-114 cells failed to react in complement fixation tests with antisera to the species-specific gs-1 antigens of feline, murine, hamster, rat, or avian C-type tumor viruses. In common with all RNA tumor viruses examined, however, the virus did contain an RNA-dependent DNA polymerase.[26]

In animal systems studied, all naturally occurring sarcoma viruses are defective[27] and require related leukemogenic virus as helpers. If the human situation is similar, one would predict that, like the leukemias[28], human sarcomas would also contain RNA that can hybridize to DNA homologous to the RNA of Rauscher leukemia virus. Eighteen of 25 human sarcoma specimens examined did contain RNA possessing homology to the RNA of the Rauscher mouse leukemia virus but not to that of the unrelated viruses causing mammary tumors in mice or myeloblastosis in chickens.[29] Further, no normal human adult or fetal tissues showed significant amounts of RNA specific to the Rauscher virus.

Malignant human breast tumors were found to contain RNA molecules that exhibit homology to the RNA of the mouse mammary tumor virus but not to that of the Rauscher leukemia virus.[30] No detectable reaction was observed between RNA from normal human breast tissue and DNA complementary to mammary tumor RNA nor were positive responses observed with any of the human leukemias and sarcomas tested.

Particles morphologically similar to the type B mouse mammary tumor virus and type C murine leukemia-sarcoma viruses have been found in samples of human milk. Both types of particles have also been found in a spontaneous mammary carcinoma in a Rhesus monkey.[31] Both the simian[32] and the human[33] virus isolates were found to contain an RNA-dependent DNA polymerase (reverse transcriptase). The viruses from human milk were also shown to contain a high molecular weight RNA which serves as a template for the enzyme.[33] The human viruses thus exhibit several similarities to known RNA tumor viruses.

Electron microscopic examination of an extended series of human milk specimens revealed a similar distribution of virus-like particles among samples from women with or without a history of breast cancer.[34] This suggests that if any one of the classes of particles observed represents an oncogenic virus related to human breast cancer, then the virus may be widely distributed. In view of the morphological similarity of the type particle designated MS-1 to B particles, the presence of antibody in human sera that partially neutralizes the

infectivity of mouse mammary tumor virus,[35] and the homology of the murine mammary tumor virus RNA to the polysomal RNA extracted from human breast tumors,[36] the possibility of the MS-1 particles being a human breast cancer virus is very strong. Alternatively, the MS-3 (C type) particles found most frequently in human milks may be the particles in some way related to human breast cancer and MS-1 particles may be merely "passenger" agents. This situation would be analogous but opposite to mammary cancer in mice, where B particles are abundant and a very few C particles can be found. The finding of C type particles associated with rat and monkey mammary tumors supports such a hypothesis.

The finding of virus-like particles in milk from women without a history of breast cancer is consistent with the concept that the genetic information for tumor virus replication and for tumor production is present in all cells but is expressed only when triggered by radiation, chemicals, other viruses, etc. Such a hypothesis is at present impossible to test but it is supported by a now considerable body of evidence. Normal embryonic tissues of some "leukosis-free" chicken strains have been shown to possess an antigen which is indistinguishable from the group-specific (gs) antigen of avian tumor viruses.[37] Subsequent work indicates that normal cells from gs^+ chickens contain at least the partial genome of an avian tumor virus. Induction of virus release has now been achieved in both gs^+ and gs^- cells after treatment with ionizing radiations or chemical mutagens and carcinogens.[38] This indicates that the viral genome is present in gs^+ and also in gs^- cells. Therefore, the chromosomal locus which controls the presence of natural gs antigen in chicken cells does not represent the viral genome itself but regulates its expression in normal cells. The results support the hypothesis that genomes of RNA tumor viruses exist as natural components of normal cells.

Tritium-labeled 70S RNA's from several avian tumor viruses hybridize from 3 to 10 times more with DNA from uninfected chicken cells than with DNA from E. coli, calf thymus, or baby hamster kidney cells.[39] After infection of chicken cells with RSV (RAV-1), SR-RSV, or RAV-2, the amount of 70S avian tumor virus ^{3}H-RNA hybridized increased 1.6 times. There was no difference in the hybridization of 70S RNA from RSV (RAV-1), RAV-1, or RAV-60 with DNA either from chicken cells that contain RAV-60 in a nonreplicating form or from chicken cells that do not appear to contain RAV-60. These results indicate that both types of uninfected chicken cells contain DNA that is complementary to RNA from several avian tumor viruses and that the amount of complementary DNA increases in such cells after infection with an avian tumor virus. The RNA's of genetically different avian tumor viruses appear to have indistinguishable base sequences by this technique.

The effect of unlabeled cellular DNA upon the reassociation kinetics of labeled double-stranded DNA made by DNA polymerase from avian tumor viruses was used to measure virus-specific nucleotide sequences in chick cells.[40] Multiple copies of these sequences were found with equal frequency in normal chick cells, in chick cells transformed in culture, and in Rous tumor cells. Copies were also present equally in cells harboring chick helper factor and gs antigen, and in cells lacking those characteristics. A similar number of viral genes

(about 15 copies) were found in chick cells regardless of the degree of expression of viral information. Quail DNA also contains multiple copies of these sequences, but no copies were detected in HeLa cell DNA or salmon-sperm DNA.

Infectious viruses capable of serial propagation have been isolated from hamsters only on rare occasions, mainly from tumors induced by murine sarcoma viruses. These viruses, termed "hamster specific", have an altered host range and do not share envelope or gs antigens with the murine C-type viruses. The available data indicate that these results may be interpreted as in vivo rescue of the murine sarcoma virus genome by a hamster-specific leukemia virus (HaLV). Evidence has now been obtained that the latent or repressed HaLV genome may be widely disseminated in hamster colonies, but can be activated and isolated only under certain conditions. Cell cultures of Syrian hamster embryo were treated for 7 days with selected chemical carcinogens; certain cultures were morphologically transformed by three different chemical preparations and yielded cell lines that subsequently produced malignant tumors in hamsters.[41] Although the cell lines were negative for infectious virus before inoculation into animals, hamster-specific C-type RNA virus was isolated from tumors or from cell lines derived from the tumors.

Tumors and virus-related antigens induced by 3-methylcholanthrene (MCA) were observed in 16 genotypically different strains of mice having differing natural expressions of C-type RNA tumor virus genomes.[42] The MCA-induced tumors contained complement-fixing antigens reactive with rat antisera selected for high-titered reactions to the gs antigens of the C-type RNA tumor virus. Since the corresponding normal tissues did not reveal gs antigen, the concurrent activation, or derepression of phenotypic expression of viral-related antigens in many of the tumors, suggests that oncogenes of C-type RNA virus genomes have served as specific determinants of the induced cancers.

In human cells, nucleic acid hybridization techniques have revealed that Epstein-Barr (EB) viral DNA comprises between 0.06 and 1.6% of the total DNA of Burkitt lymphoma cells and established human leukocyte cell lines from both leukemic patients and normal controls.[43] EB virus has many characteristics of an oncogenic virus and is widely believed to play a role in the development of Burkitt's lymphoma and nasopharyngeal cancer in humans.

That some form of stable interaction between virus-specified DNA and the host cell genome is necessary for cell transformation can be inferred from several lines of evidence. In the case of RNA tumor viruses, this interaction is believed to be made possible by the synthesis of a DNA molecule on the viral RNA template. The defectiveness of a strain of Rous sarcoma virus, RSV α(0)41 may be due to its very low activity of the enzyme which catalyzes this reverse transcription.[44] Despite its deficient reverse transcriptase, the mutant had a normal level of DNA-dependent DNA polymerase activity, suggesting that these two enzyme activities reside in physically distinct molecules.[44] However, analysis of the properties of a soluble DNA polymerase from the Prague strain of Rous sarcoma virus indicates that both the RNA-dependent and DNA-dependent activities are due to the same enzyme.[45] Neither of these studies excludes the possibility that different sites on the same enzyme or different factors might specify the RNA-or DNA-dependent activities.

In addition to the RNA-and DNA-dependent polymerase activities, virions of RNA tumor viruses contain a DNA endonuclease. Virions of the Schmidt-Ruppin avian sarcoma virus have now been shown also to contain both polynucleotide ligase and DNA exonuclease activities.[46] These enzymes complete the machinery necessary to transfer information from RNA to double-stranded DNA and to integrate this molecule into the host DNA, but further experiments are required to establish that this is their real biological function.

Analysis of the initial product of the endogenous RNA-dependent DNA polymerase of both Moloney leukemia virus and avian myeloblastosis virus indicates that it consists of a covalently bonded DNA-RNA molecule.[47] The RNA in the product presumably acts as a primer for the endogenous reaction. Experiments utilizing the inhibition of the polymerase reaction by dideoxythymidine triphosphate indicate that the DNA is attached to the 3'-OH of the primer RNA.[47] One form of the mouse mammary tumor virus also appears to contain either DNA or a DNA-RNA hybrid. Mammary tumor virus particles isolated from mouse red blood cells were found[48] to contain at least some DNA, in contrast to particles isolated from milk, which appear to contain RNA only. Purified preparations of Rous sarcoma virus contain small amounts of double-stranded DNA. However, this DNA cannot be hybridized to the viral RNA but will reanneal completely with the DNA of avian cells. Extensive substitution of bromodeoxyuridine for thymidine in "viral" DNA does not photosensitize the biological activity of the virus. These observations and supporting data indicate that the DNA associated with Rous sarcoma virus is derived in random fashion from the DNA of the host cell and is probably devoid of any function in the life cycle of the virus.[49] A report that DNA is associated with the plasma membrane of human diploid cells[50] raises the possibility that the DNA found in oncornaviruses is an envelope constituent, acquired when virions are released from the host cell.

Host cell mitochondrial functions appear to be involved in transformation by both DNA and RNA tumor viruses. Infection of confluent monolayer cultures of African green monkey kidney cells with SV40 was found to result in an enhanced synthesis of both nuclear and mitochondrial DNA.[51] With monkey BSC-1 cells, where SV40 infection does not result in a stimulation of nuclear DNA synthesis, no stimulation of mitochondrial DNA synthesis was observed. SV40 infection of mouse 3T3 cells also stimulated nuclear and mitochondrial DNA synthesis in these experiments. Polyoma virus has previously been found[52] to stimulate mitochondrial DNA synthesis in confluent monolayer cultures of mouse cells. The effects of mitochondrial inhibitors on Rous sarcoma virus focus formation and virus production in chick embryo fibroblast cultures suggest that synthesis of some protein on mitochondrial ribosomes may be required for the production of infectious RSV.[53] The recent demonstration that ethidium bromide inhibits reverse transcription of murine leukemia virus *in vitro*[54] could account for the suppression of focus formation, but the normal levels of infectious virus observed would not be expected if provirus formation were blocked.

Oncogenic DNA viruses cause a change in the cell surface which is reflected in an increased agglutinability by wheat germ agglutinin (WGA). Virus-directed or -induced synthesis of host DNA is required for this change to occur according to results obtained when several oncogenic DNA viruses were tested in mouse

or monkey cell cultures.[55] The surface change occurred in all cell lines in which cell DNA synthesis was induced but did not occur in the line in which it was not induced nor did it occur in the presence of inhibitors of cell DNA synthesis.

Concanavalin A (ConA) is another plant agglutinin which is bound to transformed cells but not to normal cells under most conditions. Vaccinia virus, early in its replicative cycle, induces a surface change in normal rabbit kidney cells which renders them capable of being agglutinated by concanavalin A.[56] This Con A binding seems not to be a transistory phenomenon since infected cells were shown to be capable of binding ConA throughout the replicative cycle. Induction of the ConA receptor in this system requires early protein synthesis but not viral or cellular DNA synthesis. It is unknown whether ConA binds to "early" structural or nonstructural viral glycoproteins or to normal preexisting cellular ConA receptors which are in some manner unmasked as a result of vaccinia virus infection.

CANCER BIOLOGY AND BIOCHEMISTRY

A commonly observed characteristic of tumor cells is the presence of proteins not normally found in corresponding normal adult tissue. Numerous studies have now demonstrated that many of these "abnormal" proteins are actually constituents of normal fetal tissues. In one such study,[57] for example, tumors of rat liver, kidney, skeletal muscle, and mammary gland contained mainly the fetal types of three enzymes. When there was displacement of adult by fetal type isozyme, however, it was not always an absolute change-over but often a change in proportions. These results, in conjunction with the different total activities of the tumors, are interpreted as indicating independent rather than reciprocal control of the fetal and adult types of enzymes. Altered transcription of DNA, especially derepression of genes with transcription of RNA's not made in normal tissue, has often been assumed to be the explanation for the abnormal phenotype of tumor cells. However, hybridization has shown no gene families active in rat hepatomas that were not active in normal rat liver.[58] The next step in the gene-to-protein sequence is the transport of RNA from its site of synthesis in the nucleus to its site of translation in the cytoplasm. Selective transport of RNA is a control mechanism unique to eukaryotic cells, and its loss may be necessary to allow the phenotypic expression of the malignant state in which cells exhibit a pattern of growth more appropriate to prokaryocytes. Investigation of RNA's in azo dye-induced primary rat hepatomas showed that RNA base sequences restricted to the nucleus in normal rat liver are found in the cytoplasm in these tumors.[59] This migration of RNA out of the tumor cell nucleus is an early effect of the carcinogen, appearing in a significant fraction of the liver cells after the animals were fed a diet containing 0.06% 3'-methyl-4-dimethylaminoazobenzene for only 6 days. Liver cells of rats fed the noncarcinogenic analog aminoazobenzene have nucleus-restricted RNA equivalent to that of normal liver. Some of the normally restricted RNA's are present in the cytoplasm of 19-day fetal liver and of regenerating liver 2.5 hr after 30% hepatectomy. No RNA's were found to be restricted to the nucleus in the tumor cells. This alteration in RNA transport demonstrated in this report provides an alternative to the hypothesis of gene derepression to explain some of the properties of malig-

nant cells. The translation of RNA's that are normally sequestered from the site of protein synthesis could account for the production of proteins not found in comparable adult tissue.

Modification of transfer RNA's is another possible mechanism for altering gene expression. Several chemical carcinogens appear preferentially to attack the tRNA fraction of the target tissue. Modification of guanosine by 2-acetylaminofluorene (AAF) appears to be associated with rotation of the base about the glycosidic bond from "anti" to "syn" followed by a stacking interaction of AAF with an adjacent base.[60] Molecular models show that these conformational changes would prevent normal base pairing between the AAF-modified base, as well as its neighbors, with bases in a complementary strand of nucleic acid.[61] The distortion produced would extend over a distance of several nucleotide residues. Guanosine residues which are in single-stranded regions are likely to be more susceptible to AAF modification than those in double-stranded regions. This may in part explain the specificity of modification observed in studies with $tRNA^{fMet}$.[61] Covalent attachment of benzo(a)pyrene to guanosine residues in nucleic acids has recently been demonstrated.[62] If this modification also is shown to be a substitution on position 8, then the proposed model[61] may extend to polycyclic carcinogens. Thus the conformational changes described with AAF may provide a unifying principle for understanding the effects of aromatic amine and polycyclic hydrocarbon carcinogens on the structure of nucleic acids.

Tyrosyl-, histidyl-, asparaginyl-, and phenylalanyl-tRNA's from a spectrum of Morris hepatomas were compared with the same respective aminoacyl-tRNA's from normal rat liver by means of MAK column chromatography.[63] The results provide strong evidence that the tRNA population of certain tumors differs qualitatively from that of the normal tissue from which the tumor was derived. The alterations cannot be attributed simply to the rapid growth of the tumor cells since the altered tyrosyl-tRNA seen in the Novikoff hepatoma and hepatoma 3924A is not seen in the rapidly growing 3683F or in regenerating liver. It is noteworthy that the hepatomas which are generally least deviated from liver with respect to their karyotype, histology, growth rate, and enzyme patterns, namely hepatomas 9121 and 9098, exhibit changes only in their phenylalanyl-tRNA profiles, whereas new species of tyrosyl-, histidyl-, and asparaginyl-tRNA's are seen only in those hepatomas which are generally representative of advanced states of malignancy, namely hepatoma 3924A and Novikoff hepatoma. Since hepatomas 3683F and 5123C do not fit exactly into either category, the possibility that specific changes in tRNA reflect the biochemical individuality of each tumor cannot be ruled out. The molecular basis for the chromatographic alterations observed might be (a) a change in primary sequence not related to secondary modifications; (b) a change in secondary modification, such as overmethylation, of tRNA species normally present in liver; or (c) a change in the relative abundance of isoaccepting species of tRNA's normally present in liver.

Transfer RNA populations from a variety of solid human tumors have also been compared with tRNA's from normal tissues by MAK and reverse phase chromatography.[64] Most tumors examined contained tRNA species chromatographically indistinguishable from those found in normal human tissues. However, 1 of 3 breast

carcinomas contained tyrosyl tRNA which eluted from MAK and reverse phase columns at a higher salt concentration than the bulk of the corresponding RNA from normal tissues. This tyrosyl tRNA was chromatographically similar to that found in many cultured tumor cells. Although neither the structural basis nor the physiological significance of this alteration in tyrosyl tRNA is known, it may be significant that the change occurred in the most aggressive and rapidly growing tumor examined.

Alteration of the configuration and functional properties of tRNA's by modification of minor nucleotides might result in synthesis of anomalous histones or acidic chromatin proteins which, in turn, might lead to chance derepression of certain segments of the genome, giving in a few cells a particular array of expressable information.[65] The attachment onto a polymer of a simple methyl group, for example, may be the most important process by which nature can differentiate without losing genotypic integrity.[65] Abnormal methylation has been found in DNA as well as in the tRNA's. During base ratio and nearest-neighbor analyses of DNA isolated from cultured human leukemic lymphoblasts, an anomalous base identified as 5-methylcytosine, was repeatedly detected.[66] No significant quantities of this methylated base were found in similarly prepared lymphocyte DNA from a normal donor or from a patient with infectious mononucleosis. On the other hand, histones in human leukemic lymphoblasts and in lymphocytes from patients with infectious mononucleosis may be similar if not identical.[67] Whole histones from human leukemic cells and infectious mononucleosis lymphocytes, in addition to having similar amino acid compositions, induced essentially identical degrees of inhibition of DNA-dependent RNA synthesis in WI-38 human fibroblasts.[67] Histones from normal human lymphocytes inhibited RNA synthesis to a significantly lesser degree.

Hormonal regulation of gene activity has been extensively studied in both normal tissues and tumors. Estrogen-dependent tissues contain characteristic estradiol-binding macromolecules (estrogen receptors) with which the hormone interacts. On entering uterine cells, estradiol associates with the 4S binding unit of an 8S extranuclear receptor protein, activating it to undergo a temperature-dependent transformation to a 5S form. The transformed hormone-receptor complex migrates to the nucleus where it associates with an acceptor site in the uterine chromatin to induce an acceleration of biosynthetic processes. Exposure to the estradiol-receptor complex of uterine cytosol has been shown to increase the RNA synthetic capacity of purified rat uterine nuclei but not of liver or kidney nuclei.[68] This stimulation is effected only by complex in which the binding unit of the receptor has undergone estrogen-induced conversion from the native (4S) to the transformed (5S) form. The results suggest that an important function of estrogenic hormone is to promote transformation of the receptor protein to an active form which can enter the nucleus and stimulate RNA synthesis. A transplantable rat mammary carcinoma (R3230AC) which does not regress after ovariectomy has now been shown to have a markedly reduced amount of cytoplasmic estrogen receptor.[69] Cytoplasm from the tumor fails to interact with estradiol sufficiently to permit estradiol binding to tumor chromatin. This defect can be corrected <u>in vitro</u> by substituting cytoplasm, containing the binding protein, from rat uterus, thus permitting estradiol binding to tumor chromatin. The results indicate that the hormonal autonomy of this carcinoma is due to a lack of receptor and not

to the inability of estradiol to interact with the cell genome. The results suggest that chromatin of these tumor cells possesses the capacity for extensive interaction with the estradiol-receptor complex.

A rat hepatoma cell line has also demonstrated an apparent deficiency in a cell regulator-binding protein. HTC (hepatoma) cells have no detectable adenyl cyclase activity or cyclic AMP (cAMP) when grown in suspension culture and, unlike normal liver, respond minimally to exogenous cAMP. Although protein kinase activity in HTC cell cytosol is 3 times higher than liver, cAMP results in a 5-6 fold greater stimulation of kinase activity in the latter.[70] HTC cytosol binds much less cAMP than liver. Since liver cAMP-binding protein inhibits HTC kinase, it appears that the altered kinase regulation in HTC cells is largely related to the deficiency of cAMP-binding protein.

Dietary feedback control of enzyme synthesis is another normal cell regulatory mechanism which has been reported to be impaired in certain tumors. In normal liver, dietary cholesterol specifically inhibits those reactions in the cholesterol biosynthetic pathway responsible for conversion of β-hydroxy-β-methylglutarate to mevalonate. All hepatomas examined (in man, rodents, and fish) have shown complete unresponsiveness to this inhibitory effect of exogenous cholesterol. Several rat hepatomas in which this apparent defect was observed have been examined[71] for presence of the diurnal rise in β-hydroxy-β-methylglutaryl CoA reductase activity induced in normal liver by feeding. Two of the three tumors studied were found to exhibit such an increase. Persistence of diurnal rhythm and absence of regulation after cholesterol feeding in hepatomas indicate that there are at least two different control mechanisms for this enzyme.

Evidence for deletion of the cholesterol feedback system from hepatomas has been based entirely on *in vitro* measurements in tissue slices or homogenates. The influence of hepatomas on sterol metabolism has now been studied in the intact rat.[72] Hepatoma-bearing rats consistently demonstrated an apparent loss of the cholesterol feedback system. The tumors retained only minor amounts of the sterol they synthesize, releasing over 90% of such endogenous sterol into the circulation. Loss of responsiveness to dietary cholesterol was demonstrated *in vivo* in 2 minimal deviation hepatomas and in one highly malignant adenocarcinoma of hepatic origin. The concept of an intracellular deletion of the cholesterol feedback system, however, requires reevaluation. There is now evidence that the defect may exist at the periphery of hepatoma cells rather than being intracellular. Several transplantable rat hepatomas have been shown to be inefficient in taking up isotopically labeled cholesterol from the blood stream after intestinal absorption.[73] The tumors thus do not accumulate cholesterol or cholesterol esters after prolonged feeding of cholesterol. While this finding does not rule out the possibility of an internal cellular derangement in the feedback mechanism, it provides an adequate explanation of the observed phenomena without postulating such an additional cellular lesion. Moreover, it illustrates the complex ramifications for cell metabolism which can result from a change in the cell surface.

A variety of transformed cells have been shown to exhibit surface changes which alter their agglutinability by certain lectins. The association between agglutinability by concanavalin A (con A) and malignancy *in vivo* was investigated using transformed hamster cells.[74] Increased tumorigenicity was found from 1-4 days after subculture, and the more agglutinable cell lines were more tumorigenic. The sites for con A on the surface membrane contain a component B that binds con A molecules and a component A that determines agglutination. B is active in normal and transformed cells, whereas A, which is associated with a metabolic activity, is in an active form only in transformed cells. A can be activated by trypsin, and is not asssociated with sites for the wheat germ and soybean agglutinins. Non-agglutinating transformed cells become agglutinable and more tumorigenic after treatment with trypsin. Trypsin-treated normal cells were also agglutinable but they did not produce tumors.

Normal nonagglutinable embryonic chick fibroblasts in confluent cultures also can be made agglutinable by Con A by treatment with any of several inhibitors of protein synthesis.[75] Normal (low level) agglutinability reappears within 6 hours after removal of the inhibitor. Similarly treated cultures of growing cells failed to become more agglutinable. The results suggest that molecules which maintain the normal structure of the cell surface must be replaced continually when cell growth is contact-inhibited. Cell aggregation factors released from chick embryo and several mammalian cell cultures have been identified as acid mucopolysaccharides.[76] Cell-to-cell binding by acid mucopolysaccharides may regulate contact inhibition of movement and growth *in vitro* and related *in vivo* phenomena.

The hypothesis that cyclic AMP plays some role in the control of cellular growth is supported by three observations: (a) cyclic AMP levels are lower in transformed cells than in normal cells; (b) agents which increase cellular cyclic AMP levels suppress growth; and (c) agents which decrease cellular cyclic AMP levels stimulate growth. The possible involvement of the cell surface in the action of dibutyryl cyclic AMP is suggested by changes in the agglutinability of dibutyryl cyclic AMP-treated transformed cells.[77] Dibutyryl cyclic AMP restores contact-inhibited growth to some transformed cells in culture. This exogenous dibutryl cyclic AMP may compensate for an inherent deficiency of cyclic AMP in transformed cells. The steady state levels of cyclic AMP in normal density-dependent mouse fibroblasts were recently shown to be twice as high as those in the corresponding viral and spontaneous transformants examined.

HOST-TUMOR INTERACTIONS

Attempts to improve understanding of normal immunological mechanisms and to apply this understanding to control of malignant disease have focused on the identification and separation of the various components of the lymphoreticular system.[79] Present data suggest a two-component lymphoid system consisting of bone marrow-derived stem cells that differentiate into two types: B lymphocytes responsible for humoral antibody synthesis and T lymphocytes responsible for cell-mediated reactions (homograft rejection, delayed hypersensitivity, graft-versus-host reactions and tumor immunity) and for cooperation with B lymphocytes in some antibody responses.

Recent basic studies show that immunologic memory can be expressed by both T and B cells.[80] Cooperation between T and B cells also plays an important role in the induction of immunological tolerance. It now appears that tolerance can be transmitted from cell to cell. A recent study[81] demonstrates that adoptively transferred thymus-derived lymphocytes made tolerant to sheep red blood cells were able to transfer non-reactivity and prevent the cooperation of normal thymocytes with bone marrow precursors of antibody-producing cells. Immunologic tolerance has been proposed to explain the life-long persistence of certain viruses in infected mice. The absence of classical immunologic tolerance has been demonstrated[82] in AKR mice naturally infected in utero with Gross leukemia virus. Glomerular deposits of Gross antigen-Gross antibody complexes were found in some mice by 3 months of age and increased with time. Ten percent of these mice apparently died from this complex disease, indicating that clinical tolerance to Gross antigens was nonexistent. Another study[83] has demonstrated that mice neonatally infected with the mammary tumor virus (MTV) can produce detectable quantities of humoral antibodies that react with the MTV virion following their recovery from transient immunosuppression induced by antithymocyte serum.

Phytohemagglutinin (PHA) and pokeweed mitogen (PWM) are blastogenic for T and B cells, respectively.[84] Lymphocyte transformation in response to these plant mitogens can be useful in assessing immunologic competence in the tumor-bearing host. In a study[85] of lymphocyte transformation, in response to PWM in leukemic children who had completed therapy, it was shown that during remission lymphocytes responded within normal limits to PWM in patients who had not received intensive forms of therapy, but after intensive therapy plus craniospinal irradiation had been completed, lymphocyte transformation was impaired for at least 2 months and in some cases for four or more months. In another study[86] of children with acute lymphocytic leukemia in remission, it was shown that maintenance combination chemotherapy inhibited both the primary and secondary antibody response. Serum IgG was depressed in one-fourth of all children with leukemia in remission. In vitro transformation of lymphocytes by PHA was a poor index of immunocompetence in this study since after 2 years of continuous chemotherapy, children with low IgG and suppressed antibody production had normal response to PHA. In still another study[87] of 19 patients with acute leukemia, it was shown that regardless of the status of disease, remission or relapse, these patients retained their ability to produce immunoglobulins in response to infection even though their immunoglobulin levels were reduced by therapy. Infections occurring in these children when immunoglobulins were depressed as much as 50% below pretreatment level seemed no more severe than at other times.

Several studies have shown a strong correlation between immunologic competence and prognosis after therapy. Cell-mediated immunity correlated[88] with the response to chemotherapy in patients with acute myelogenous leukemia (AML); 86 percent of patients with normal delayed hypersensitivity to an established battery of skin test antigens achieved remissions as compared with 25 to 50 percent of anergic patients. In vitro lymphocyte responses to PHA before and after therapy were studied by measuring radioactive thymidine incorporation into DNA. There was no significant change in incorporation in patients achieving remission but those not achieving remission showed a decrease from 15,000

to 9,000 cpm. Sixty percent of these patients showing delayed hypersensitivity after immunization to keyhole limpet nemocyanin (KLH) entered complete remission as compared to only 37 percent of those without this reactivity. Patients with acute leukemia and solid tumors converting from immunoincompetence before therapy to immunocompetence during therapy achieved remission, whereas those converting from immunocompetence to immunoincompetence did not. In another study,[89] it was shown that patients with metastatic solid tumors and AML who responded favorably to chemotherapy with tumor regression not only recover their cellular response 8 to 10 days after treatment but "rebound" to levels higher than pretreatment levels. This shows that the rebound with overshoot of immunologic reactivity after chemotherapy correlates with tumor regression and prognosis, and continued suppression may be an indicator of poor prognosis. Thus, serial immunologic evaluation during therapy may yield valuable data relating disease, therapy and prognosis.

Results of a study[90] of 21 cancer patients skin tested with 2,4-dinitrochlorobenzene (DNCB) and the cutaneous irritant, croton oil, suggest that failure to respond to DNCB may be caused by a defective inflammatory response rather than by faulty cellular immune recognition or responsiveness. Twelve of the 13 patients who did not react to DNCB did not have defects in delayed hypersensitivity per se, since none of them manifested an inflammatory response to croton oil. Thus, only one of the 13 nonreactors might be considered anergic to the compound. These findings demonstrate that criteria independent of skin testing may be required to assess the competence of the cellular immune system.

The delayed hypersensitivity reaction has been implicated in the body's defense against cancer and the production of migration-inhibitory factors is a characteristic of the delayed hypersensitivity response. It has been shown[91] that human tumors either contain or produce a substance which inhibits in vitro leukocyte migration. Some preparations were cytotoxic to both autogenous and allogeneic leukocytes. In five breast cancer patients studied the one with the most advanced disease demonstrated the greatest inhibition of leukocyte migration. The growth of heterologous hepatoma cells was not inhibited by some preparations that inhibited leukocyte migration. Thus, both cytotoxic and migration-inhibiting substances can be liberated by tumor leukocyte interactions. These findings suggest that delayed hypersensitivity may operate as a defense mechanism early in the natural history of cancer but that as the cancer becomes advanced certain components of the reaction, specifically, inhibition of leukocyte migration, become detrimental.[92]

Hodgkin's disease patients demonstrate defective cellular immunity, mediated by lymphocytes of thymic origin. A child with stage I disease presented with anergy at diagnosis.[93] The child had a normal immune response following therapy and a return of anergy with relapse. The humoral immune system was intact during a period of inactive cellular immunity. Antigen recognition for the cellular immune mechanism was intact, but not demonstrable, during a period of inactive cellular immunity. The recognition mechanism persisted, and could deliver information, after cellular reactivity had returned to normal. These findings suggest that the immunodeficiency of Hodgkin's disease is caused by the disease process. Multiple myeloma is associated with an immune deficiency state. Patients with multiple myeloma have an impaired

humoral and cellular immunity in response to keyhole limpet hemocyanin (KLH).[94] When compared with controls, the patients showed a prolonged induction time for IgM antibody formation and a more rapid switch from IgM to IgG production. Patients with myeloma also had reduced *in vitro* lymphocyte responses to streptolysin-O and vaccinia. Immune function of plasmacytoma patients, however, was similar to that of controls.

Malignant transformation of cells is often associated with alterations in the cell membrane and the appearance of substances of tumor origin which possess unique antigenic structure. These antigens and the antibodies to them have been demonstrated in a wide variety of cancers: Recent studies[95] have demonstrated tumor-specific, cell-mediated immunity to primary intracranial neoplasms, both well differentiated and anaplastic. Some studies suggested antigenic cross-reactivity between glioblastoma cells from different patients and between glioblastoma and melanoma cells. Immunofluorescence studies[96] have demonstrated that the sera of some patients with breast carcinoma contain antibodies which react with antigens in cells of breast carcinoma grown in tissue culture as well as with cells of cultures derived from fibrocystic disease and osteosarcoma. Sera of a small number of patients with osteosarcoma reacted with cells of fibrocystic disease and breast cancer cells.

In vitro studies demonstrating immunity to antigenic tumors by inhibition of tumor colony formation probably assay the activities of both T and B cells. Investigations are continuing to clarify the nature of the demonstration that experimental animals and human patients have immune lymphocytes that can react against their tumor cells as well as serum factors that can "block" and "unblock" these reactions. Experiments[97] in mice suggest that the serum blocking factor is an antigen-antibody complex capable of binding to the target cells and/or reacting with immune lymphocytes. Another study[98] suggests that desensitization of sensitized lymphocytes by soluble tumor-specific or histocompatibility antigens may be responsible in part for the specific abrogation of sensitized lymphocyte activity by serum from tumor-bearing animals. Immunotherapeutic procedures capable of abrogating blocking activity such as passive inoculation of "unblocking" antibodies from animals or patients, or the induction of antibody formation in the tumor-bearing host may eventually become adjuncts to chemotherapy or surgery.

Various approaches to immunotherapy are being evaluated in experimental animal models before extensive application to human cancer. Established methylcholanthrene fibrosarcomas in mice have been completely rejected following vaccination with living tumor cells treated with *Vibrio cholerae* neuraminidase (VCN) plus mitomycin C in combination with BCG.[99] Modification of graft-versus-host disease (GVHD) has been achieved by VCN treatment of donor cells.[100] VCN treatment appears to render cells less tolerogenic than normal so that mice do not become tolerant and the GVHD cannot develop. In another study,[101] immunization with chemically modified lymphoma cells has produced immunity in mice against a challenge of 6C3HED tumor cells. Tumor cells modified by iodoacetamide, iodoacetate, and N-ethylmaleimide protected fully and p-hydroxymercuribenzoate gave partial protection. No cytotoxic antibody in immune serum was demonstrable, but lymphoid cells could transfer immunity indicating cell-mediated immunity. Immunization against cancer-causing chemicals has been documented in a study[102]

where over 50% tumor inhibition was achieved by immunization of rats with a carcinogen-protein conjugate prior to the feeding of 2-anthrylamine. As another approach, a technique[103] has been developed which may provide a means for inducing immunologic tolerance in vitro. Blastogenic response of lymphoid cells toward any individual's transplantation antigens can be deleted by the addition of tritiated thymidine of high specificity during incubation in mixed leukocyte culture. After the immunocompetent clones which responded to histocompatibility antigens have been destroyed, the remaining population of cells can still respond to unrelated antigens. An adoptive immunotherapy for cancer exploiting this capability can be considered.

Vitamin A may modify the tumor response to radiation via a cell-mediated effect. Injection of vitamin A has been shown[104] to cause a significant reduction of 15-20% in the radiation dose required to control an antigenic fibrosarcoma in mice. The drug did not increase the severity of normal tissue reactions after irradiation. Following irradiation, radiation-damaged cells prior to their lysis may compete with surviving cells for a limited immune rejection response. It is suggested that vitamin A may cause more rapid lysis of radiation-damaged cells thus rendering the remaining viable cells more susceptible to the immune defenses of the body.

A recent study[105] has presented histologic evidence for immunological surveillance during skin carcinogenesis. Using the system of isograft-promotion of MCA-treated skin in mice it was demonstrated that focal microscopic lesions exhibiting mononuclear cell infiltration were commonly found only in immunologically competent hosts during the latent period of papilloma development. Common histological features of microscopic inflammatory foci at known papilloma regression sites included dermal keratin debris, lymphocyte exocytosis, and mast cell degranulation associated with epithelial pyknosis. The evidence strongly suggests that the inflammatory foci represent the remnants of immunologically destroyed papillomas.

The tumor angiogenesis factor (TAF) isolated from human and animal tumors is mitogenic to endothelial cells and stimulates rapid formation of new capillaries in animals. A quantitative technique for measuring the degree of angiogenesis in tumor has been developed.[106] The histologic grading system is based on 3 parameters of endothelial regeneration: vasoproliferation, endothelial cell hyperplasia, and endothelial cytology. Solid neoplasms could be classified according to the extent of angiogenesis along a spectrum ranging from the "endothelial-poor" tumors such as chondrosarcomas, to the "endothelial-rich" tumors such as glioblastomas. Anti-angiogenesis may provide a powerful adjunct to cancer therapy. One approach to the initiation of "anti-angiogenesis" currently in progress is an attempt to produce antibody against TAF.[107]

Blood vessel invasion previously has been shown to be of prognostic importance in several types of cancer. Blood vessel invasion was seen in 31 of 74 cases of bladder cancer evaluated.[108] The five-year survival in this group was 29% in contrast to 51% in the 43 cases without blood vessel involvement. Increased frequency of blood vessel invasion was found in tumors of higher grade and with deeper penetration of bladder wall. In a study[109] of 243 patients with

squamous cell carcinoma of the nasopharynx, tonsillar fossa, and base of the tongue, it was shown that the frequency of distant metastases following local control did not increase with the size of the primary tumor. Patients with advanced nodal disease had a higher frequency of distant metastases at five years than did those with early nodal disease. Contrary to accepted opinion the location of neck disease did not influence the frequency of distant metastases. Results of a retrospective study[110] of 74 patients who had therapeutic oophorectomy for advanced breast cancer showed that patients who had ovarian metastasis had higher rates of objective response than those who did not (36% vs. 11%). In addition, patients with ovarian metastasis had a longer disease-free interval and better survival after mastectomy. Survival of patients whose ovaries had corpus luteum was much better at the 4th and 5th years after mastectomy.

Since murine sarcomas induced with methylcholanthrene rarely metastasize, artificially induced metastases have been studied[111] to evaluate the effect of immune status on metastatic growth. Local or regional immunological, anatomical, and physiological differences appear to be factors that determine where artificially induced metastatic tumors may develop. It has been shown that host resistance following sensitization was expressed most strongly in the liver and lungs and less strongly in the s.c. and i.p. locations. Sensitization by living tumor implants surgically removed after a period of growth left the hosts more resistant than the mice with sensitizing implants that had been killed by local irradiation. Immunotherapy consisting of s.c. injection of killed tumor tissue after challenge in addition to presensitization resulted in stronger resistance than presensitization alone.

The controversy persists over the role of the regional lymph nodes in patients with breast cancer. Data from one study[112] demonstrate in an inbred mouse strain that tumor immunity resides mainly in the spleen and very little can be demonstrated in regional lymph nodes. Data from patients with breast cancer showed that those in whom regional nodes were removed as well as those in whom the nodes were left intact, failed to respond to ipsilateral injections of tetanus toxoid, suggesting that the regional lymph nodes are immunologically incompetent. Another study[113] utilizing a syngeneic mouse host-mammary tumor system, indicates that the regional lymph node is important in the development of tumor immunity. In the absence of regional lymph nodes, no apparent sine-comitant nor concomitant immunity developed despite the presence of a growing immunizing tumor for as long as 28 days. These findings minimize the role of the bloodstream in host sensitization to tumor and emphasize the importance of a direct lymphatic communication to nodes for the optimal development of delayed hypersensitivity or transplantation immunity. It is possible that the tumor antigen from the very onset of tumor growth disperses through the host in such a fashion that the concentration in lymph nodes required for an immune response is delayed or fails to occur. These findings provide another explanation for de novo development of an antigenic tumor in an immunologically normal host. These studies emphasize the importance of randomized clinical trials to evaluate the importance of the regional lymph nodes in breast cancer.

EPIDEMIOLOGY

Breast cancer risk appears to be associated with indices such as familial tendencies, endocrine and metabolic processes, and genetic, socioeconomic and viral factors. Results of a genetic study[114] of familial breast cancer point to the possibility of at least two types of breast cancer, one characterized by an early onset, a high frequency of bilateral disease, and a tendency to occur in individuals of blood type O; and a later type associated with unilateral involvement and possibly occurring more frequently in blood type A individuals. A genetic difference between these two types was suggested since the risk of breast cancer developing in the relatives of patients with the bilateral type was significantly higher than that in relatives of patients with the unilateral type. There was the added possibility of another type of breast cancer, a sporadic type occurring in some individuals with no family history of the disease.

It has been suggested previously that estrogen ratios during the reproductive years are related to breast cancer in later life. Specifically it appears that the non-carcinogenic, impeded fraction (estriol) partially inhibits the action of the carcinogenic, unimpeded fractions (estrone and estradiol). Determination of estrogen profiles in a series of Asian and North American women supports this hypothesis.[115] The proportion of estriol among the three estrogens both in follicular and luteal phase urines was considerably higher for Asian women, where breast cancer rates are low, than for North American women, where rates are high. This difference in estriol ratio was distinct among the 15-19 age group and slight among the older 35-39 age group.

The epidemiologic features of breast cancer in Taiwan are very similar to those in Japan.[116] The Chinese women have the same low rates of this disease that are seen in Japanese and certain other Asian and African populations. With respect to increased risk associated with early menarche, late menopause, and increased weight, the findings are consistent with those both in Japanese and American populations. The main difference in the Taiwan findings is the strong relationship of breast cancer risk to parity and only a weak association with age at first pregnancy.

The protective effect of early menopause in breast cancer has been documented previously. Analysis[117] of a series of 3,887 patients shows that among women with menopause induced before age 35, breast cancer risk remained as low as one-third that expected 30 and more years later. Women with natural menopause at age 55 or older had twice the risk experienced by those whose menopause occurred before age 45. The risk associated with late menopause was greatest after age 70. These findings emphasize the long latent period which may elapse between etiologic events and the appearance of breast cancer, and they show that the ovarian etiology of human breast cancer is not restricted to cancers appearing during the years of active ovarian function.

An association has been reported[118] between wet-type cerumen and breast cancer rates in diverse populations. Cerumen exists in two phenotypic forms, wet and dry, which are controlled by a pair of genes in which the allele for the wet type is dominant. The homozygous recessive dry type is highly prevalent in Mongoloid populations in Asia and in American Indians. The wet type pre-

dominates in Western Europeans, and in Caucasian and Negro Americans. The study indicates that wet-type cerumen is found in Japanese women with breast cancer more often than in healthy Japanese women. As one moves westward from Asia to Western Europe, the increasing mortality rate for breast cancer is inversely related to the genetic gradient of dry cerumen. Lysozyme and immunoglobulin G also are highly associated with dry cerumen. Thus, the association between wet cerumen and cancer rates may be due to the action of a pleiotropic allele, affecting the apocrine system (ceruminous, mammary and axillary apocrine sweat glands), the fat depots and other systems, which in turn may affect susceptibility to breast cancer. Epidemiologic studies[119] to identify high risk groups in breast cancer show a consistent association between increasing rate of breast cancer incidence in American Negroes and the extent of Caucasian admixture. U. S. Census data for 1966-67 show that breast cancer death rates are higher for Negro women than for Caucasian women. Mortality from breast cancer in relation to all causes of death, however, was lower in Negro women than in Caucasian women. Analysis of 1960 census data for incidence and income levels, shows that breast cancer incidence at higher income levels appears to be greater for Negroes than for Caucasians and that at lower income levels incidence is almost identical for both.

Of all the viruses known to be oncogenic in animals, the herpesviruses have been the most consistently associated with cancer in humans. There appears to be a definite association between Herpesvirus hominis (HVH) type 2 and cervical cancer but the role of the virus in the causation of the tumor is still unknown. A simple, rapid and economical model[120] has been developed for the study of the HVH type 2 infection process in organ cultures of human cervical tissues. Columnar epithelium and mucous glands of the endocervix, squamous epithelium of the ectocervix, and stromal tissues in both endo- and ectocervix all are susceptible to infection. The infection spreads more rapidly in the stromal cells than in the columnar epithelial cells. The finding of intranuclear inclusions and "ground-glass" nuclei in the same fragment of endo- or ectocervix suggests that either type of nuclear alteration may be associated with primary or recurrent infection.

A survey[121] of antibodies to HVH type 2 in women with cervical cancer and control women from two widely separated geographic areas in the U.S. has shown no significant differences between the cancer patients and controls. A similar study[122] in Uganda showed 90% of Ganda women with cervical cancer and 71% of control women of the same tribe had HVH type 2 antibodies. Among the non-Ganda women, 67% of patients and 74% of controls had antibodies. The high occurrence of antibodies in this population does not distinguish between the hypothesis that the association between the virus and cancer is etiologic or that it is a covariable of sexual activity. Another study[123] strengthens the evidence for the association of HVH type 2 with carcinoma of the cervix. A herpesvirus identical to type HVH-2 with respect to plaque morphology and microtubule formation in infected HEp-2 cells, and immunologic specificity, has been isolated from cervical tumor cells grown in culture.

A prospective seroepidemiologic study[124] of infections caused by various members of the herpesvirus group was carried out on 4,824 sera from a middle class urban population. Prevalence of complement-fixing antibodies to HVH and cyto-

megalovirus (CMV) was low in childhood and increased with age, unlike that to varicella-zoster virus (VZV) which was highest in childhood and decreased with age. Females had a higher prevalence of CMV and HVH antibodies at all ages than did males, whereas this difference was seen only in ages less than 20 with VZV antigen. There was a significant association between the occurrence of antibodies to CMV and HVH which appeared to be due to common epidemiologic factors, since there was no cross-reactivity between the antigens. Antibodies to HVH, CMV, VZV and Epstein Barr virus (EBV) were found to be lower among children 1-8 years of age with acute lymphoblastic leukemia than among controls.[125] CMV and VZV antibodies were not found in the sera of children 1-8 years of age who had leukemia, and HVH antibodies were not found in sera from leukemic children 1-4 years of age. In general, the frequency of positive sera was similar among treated and untreated patients, irrespective of their clinical status. It was suggested that infection of malignant cells with a continuous release of viral antigens may cause a consumption of humoral antibodies to the herpesviruses. Studies[126] of asymptomatic CMV infection in 42 children with acute leukemia, showed that only five patients excreted the virus in the urine, saliva or both and that there was no clinical evidence of infection. There was no suggestion that either relapse or chemotherapy was associated with dissemination of CMV infection.

A retrospective study[127] has shown a high incidence of VZV infections in patients with lymphoma. Of 1,130 patients, there were 129 cases of infections, including 21 disseminated cases. The incidence of infections in patients with Hodgkin's disease was 15.4%. A significantly increased incidence of VZV infections was found in the 150 patients with Hodgkin's disease who underwent splenectomy, although incidence of dissemination or survival in this group was apparently unchanged by the infectious complications. It was suggested that splenectomy remain as a valuable staging adjunct for Hodgkin's disease despite the apparent increased risk of VZV infection for a few months after surgery and radiation. Further studies are needed to clarify the observation[128] that a greater incidence of type 2 HVH antibody occurs in male patients with Hodgkin's disease compared with controls and that a greater incidence of the antibody occurs in patients with a mixed cellular histologic type.

Recent observations support the view that infectious agents play an etiological role in Hodgkin's disease. This is suggested by the recent finding[129] of a cluster of Hodgkin's disease with an interrelationship of 31 cases in which the disease appeared to be spread from person to person. Another study[130] has shown that cultured cells from Hodgkin's disease undergo "transformation" with the appearance of a large number of rounded, free floating cells that release into the culture medium an RNA-containing agent and a DNA-containing agent of the herpes group, though the latter's reactions are typical of neither HVH or EBV. A recent study has indicated that the risk of Hodgkin's disease under 40 years of age is almost 3 times as great for tonsillectomized persons as for those with intact tonsils. It was suggested[131] that tonsillectomy may remove a protective barrier and may have an effect similar to that of the natural involution of the oropharyngeal lymphoid tissue in late childhood. It has been proposed that Hodgkin's disease may be due to a virus of low virulence and infectivity which enters via the oral-respiratory tract portal and which is barrier-held by intact, non-involuted lymphoid tissue; that im-

mune-complex material escapes to the lymph-nodes and evokes the distinctive reaction; and that the failure to detect an agent in the nodal lesions may be due to the fact that the agent itself does not get to the lymph nodes. The habitat of the virus may be the female genital tract.

Although the etiologic significance of EBV to Burkitt's lymphoma and nasopharyngeal carcinoma has not yet been established, investigations are continuing to provide data concerning this relationship. High anti-EBV antibody titers were found[132] in American patients with carcinoma of the nasopharynx regardless of histologic type (lymphoepitheliomatous carcinoma, squamous cell carcinoma or adenocarcinoma). These findings do not support the concept that EBV replicates opportunistically in any aggregation of lymphocytes but suggest that EBV is specifically associated with nasopharyngeal carcinoma and that such a relationship may be causal.

Serially cultured human osteosarcoma cell lines[133] spontaneously develop foci of altered cells resembling the foci produced in monolayer cultures by oncogenic viruses. They also contain a common antigen demonstrable by indirect immunofluorescence with human sera. The occurrence of foci of altered cells and of a common antigen and the transmission of these two characters to indicator cells by filtrates, are all suggestive of a virus specially associated with human sarcomas, one to which the general population is widely exposed, as indicated by the prevalence of antibody. Another seroepidemiologic study[134] has shown no evidence of avian leukosis virus group-specific antibodies in 101 sera from patients with various types of leukemia.

Studies are continuing to evaluate the possible role of viral hepatitis in hepatocellular carcinoma. A survey[135] of 51 patients in the U.S. using modified counter-immunoelectrodiffusion showed only 3 patients were hepatitis-associated antigen (HAA)-positive. All three of these patients were positive for α-fetoprotein, as were 61% of the entire series. This suggests that HAA is not related to hepatoma in the U.S. In another U.S. study,[136] however, 3 of 14 patients with hepatoma had Australia Antigen (Au) and 4 of 8 tested had anti-Au. Of this series, 5 were α-fetoprotein positive. Sera from 4 of these cases were positive for either Au or Anti-Au. These results seem to support the association of long incubation type hepatitis and hepatoma.

Further epidemiologic study is needed of the relationship of dog bite and leukemia-lymphoma. The results of a study[137] of approximately 50,000 children bitten by dogs and followed for five or more years, indicate that these children do not incur significantly increased risk of developing leukemia-lymphoma. A recent eruption of leukemia in North Kingston, Rhode Island, suggests an emerging pattern.[138] A total of 14 cases of leukemia (significantly higher than expected) have occurred in an 11 year period. Nine of these cases were acute leukemia. The discovery of various associations among most of the cases suggests that inter-personal contacts of an indirect nature may have contributed to the epidemiologic pattern observed. Another study suggests that high birth weight may be associated with an increased risk of leukemia mortality.[139] Analysis by sex showed the significance was confined to females. Social class was the only other factor showing a significant association with leukemia mortality. The risk was greater for children of

higher social class. Again, the association was significant only for females. An analysis[140] of the Oxford Survey, which now covers all juvenile cancer deaths in England, Scotland, and Wales during the period 1953-68, for an association between BCG vaccination and leukemia, has demonstrated that there have never been any differences in the population of leukemia cases, other cancer, and healthy controls with records of either neonatal or later BCG vaccinations.

An epidemiologic study[141] has demonstrated that diagnostic irradiation produces an enhanced risk for adult males for both acute and chronic myeloid leukemia. The effect was more pronounced for irradiation to the trunk than to all other sites. Neither irradiated males nor females had an elevated risk for any other histologic type of leukemia. It was concluded, however, that only a small proportion of the irradiated population develops leukemia, and irradiation accounts for only a small proportion of leukemia cases. Biomathematical methods have been developed[142] to investigate problems arising in estimating from retrospective survey data the latent periods of juvenile cancers initiated by obstetric radiography. It was shown that radiogenic cases are genuine and that each diagnostic group has a characteristic radiogenic age distribution which differs from the corresponding idiopathic age distribution in being more sharply peaked and having a slightly older mean age. For radiogenic cases, each diagnostic group was shown to have a characteristic latent period.

Sunlight exposure and the relative risk of developing cutaneous and oral squamous cell carcinoma (SCC) was studied[143] in white and non-white cats. Compared with nonwhite cats, white cats had a significantly higher risk (13.4 times higher) of developing cutaneous SCC but not oral SCC. Of the total of 149 feline SCC studied, 87 were on the ears and nose, which have little or no hair covering and which are the areas of the body most exposed to U.V. rays of the sun.

Data from a case-control study[144] revealed a consistent association with cigarette smoking and occupational exposure in the etiology of lower urinary tract cancer. In addition, an apparent association of the disease with coffee drinking was demonstrated. The relative risk for coffee-drinkers as compared to non-coffee drinkers was 1.24 among men and 2.58 among women. The association was strong in the sex-age groups in which the associations of the disease with cigarette smoking and occupation were not strong. It was estimated that about one-fourth of bladder cancers among men could be due to coffee drinking and one half of the disease in women might be so caused. Although an association between bladder cancer and cigarette smoking has been demonstrated, it remains uncertain whether the association is causal. A study[145] suggests that it would be reasonable to attribute the increase in bladder cancer rates to smoking since trends in the increase of smoking and disease rates of successive birth cohorts of men and women in the United States, Denmark, England and Wales have been shown to be parallel.

The relationship of suspended particulate air pollution to mortality from cirrhosis of the liver has been under study[146] for several years in Buffalo and its immediate environs. The study area was classified into four pollutant areas by means of measurements obtained from high-volume samplers located at

21 air sampling stations. The economic status factor was controlled by grouping of census tracts into levels on the basis of median family income. Age- and sex-specific death rates among whites for cirrhosis of the liver for the period of 1959-1961, showed a graded positive association with particulate air pollution level when economic status was controlled and the association was strongest in the lowest economic groupings.

A retrospective epidemiologic study[147] of cancer of the prostate reveals the following etiologic aspects of this cancer: The low rate among Jewish men in the U.S. seems to be related to the lower rate of prostatic cancer among immigrants from Eastern Europe. U. S. Negroes have a higher incidence rate than Caucasians. No significant relationship was found in socioeconomic status, marital status, fertility, social habits, previous disease, weight, height, hair distribution, or laboratory data. The main etiologic lead to which this study could not contribute is the rarity of clinical prostatic cancer in Japan and its increase among Japanese immigrants to the U.S. This inability to determine major environmental factors may be a consequence of the possibility that prostatic cancer is hormone-related and that factors that influence these hormones are most difficult to determine by interview techniques.

A computerized procedure[148] for associating epidemiological factors with cancer of various sites has revealed the following distinguishing features of thyroid cancer patients: more tonsillectomies than other cancer patients; relationship to asthma, hay fever, eczema, neurodermatitis, contact dermatitis, and acne; and an excess of Jewish patients. Statistical analysis of these differences indicates that they are significant and provide leads for future medical investigation.

Mathematical models developed to describe biomedical phenomena are being applied to a wide variety of epidemiological studies. Biomathematical models developed for inferring the natural history of diseases have been applied[149] to endometrial cancer and have been tested against data from collaborative clinical studies. The mathematical theory adequately fitted the empirical facts from the clinical study. Deep mathematical models also have been applied[149] to the construction of predictive research designs of large scale collaborative clinical trials of new drugs and other therapeutic innovations. Another model[151] has been developed for planning the duration of long-term survival time studies designed for subject accrual by cohorts. Data based on a mathematical model[152] developed to describe the effect of length sampling bias on labeled mitotic index waves indicates that cells which have longer DNA synthesis periods have a greater probability of being labeled.

DIAGNOSIS

The synthesis of a wide variety of aberrant proteins is associated with malignancy and often determination of these markers is of value in diagnosis and in monitoring therapy. The value of the fetal antigens in diagnosis and prognosis continues to be evaluated. Alpha-feto-protein (AFP), a normal fetal serum alpha-1 globulin, is associated with primary carcinoma of the liver.

The finding[153] of AFP in the serum of a case of gastric carcinoma metastatic to the liver confirms that the globulin can occur without demonstrable hepatoma. The only nonhepatic tumors found so far with circulating AFP have come from the upper gut or very undifferentiated teratoblastomas. A fetal gut antigen (FGA) which differs from other gut antigens has been found[154] in 87% of gut extracts from human fetuses, 30% of extracts of gastrointestinal tract carcinoma and 8.7% of normal gut tissue. This globulin was also found in an extract of ulcerative colitis tissue and in the mucosa overlying a carcinoma of the rectum. It was not found in the serum of patients with gastrointestinal tract tumors.

The carcinoembryonic antigen (CEA) found to be diagnostic for digestive tract cancers has been evaluated in an enlarged series[155] and shows a 72% positivity. Serial estimations of CEA appear to be valuable in the assessment of the patient following resection of cancer of the colon. Twenty-three (88%) of the expanded series of 26 patients with cancer of the pancreas had positive assays. Approximately 50% of selected patients with severe active alcoholic cirrhosis had positive assays. Only one patient out of 41 with ulcerative colitis was found to have an elevated positive assay. One of 15 patients with benign polyps had a positive test and his assay remained positive after resection of the polyp. None of 40 normal controls have had positive assays. A glycoprotein immunologically identical with CEA has been extracted in small quantity from normal and cirrhotic livers.

New observations[156] on the Regan isoenzyme, a placental-type alkaline phosphatase, that occurs in approximately 14% of cancer patients, reveal that it also occurs in nonmalignant states commonly associated with a predisposition to neoplastic development, including ulcerative colitis, familial polyposis, and cirrhosis. Regan-positive patients, in common with alcoholics, appear to have an increased incidence of blood group O with a decrease of group A, and the predominant group of patients with non-malignant hepatic disease who are Regan-positive have had acute alcoholism or Laennec's cirrhosis. Thus, there appears to be a relationship between the derepression of the cancer cell and phenotypic alterations which may be related to alcoholism. The identification of Regan isoenzyme in gonadal tumors and in precancerous states may represent a predeliction of such derepression for tissues concerned with germinal activity or with genetically determined cancer diathesis, respectively.

Serologic studies[157] of Walker 256 tumor-bearing rats have demonstrated that the host serum feto-tumor antigen is a seromucoid. High concentrations of this component were also found in the sera of rats subjected to stress and in fetal rats. Much smaller amounts were found in normal adult rat serum. It was suggested that tumor growth induces reversion to fetal type seromucoid synthesis in the host liver.

A neoproteolipid has been detected[158] by thin-layer chromatography in high-density lipoproteins from sera of patients with various types of cancer. It is not detected in sera from normal individuals. The new proteolipid has also been isolated from lipid extracts of the rat Walker 256 tumor. The presence of this neoproteolipid in blood serum appears to be specific for malignancy and may eventually provide a serological test for cancer.

The serum lipoproteins of rats are elevated during carcinogenesis of the liver induced by 2-acetylaminofluorene.[159] Increases in low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were highly significant. It was established that the elevation of HDL was not due to dietary restriction. Host serum HDL concentrations may be related to feedback control of lipoprotein synthesis in non-hepatic tumors such as Walker carcinosarcoma, mammary tumor or Ehrlich ascites tumor and lack of it in liver tumors. It has been proposed that serum HDL may be utilized by growing cells, perhaps in the formation of cellular membranes. Thus, the synthesis, secretion and concentration of host serum HDL may play a decisive role in the development and growth of tumors.

One serum polymorphism, haptoglobin, a serum α_2-globulin that binds free hemoglobin appears to be associated with some cancers. In a recent study[160] of 294 patients with reticuloendothelial malignancies and 93 melanoma patients it was shown that haptoglobin 1-1 was 1.3 times more frequent in acute leukemia patients than in controls. The Hp 2-2 type was decreased a similar amount while 2-1 showed no change. The Hodgkin lymphoma group manifested a 1.7 excess of 1-1 and a deficiency of 2-1 but not of 2-2. The haptoglobin distributions in chronic leukemia and melanoma were similar to controls. Thus, an association appears to exist between haptoglobin type and acute leukemia and Hodgkin's lymphoma.

Loss of the A, B, and H blood group isoantigens have been demonstrated previously for carcinomas of the ovary, cervix, and lung. In a study[161] of primary and metastatic carcinoma of the stomach, the antigens could not be demonstrated in 70 of 95 primary tumors and in 149 of 170 metastases. The loss of antigen paralleled the degree of anaplasia. In another study[162] these isoantigens were diminished or absent in 8 of 9 primary gastric neoplasms and absent in all metastases. The loss or retention of these isoantigens by malignant gastric epithelium bore no consistent relationship to the degree of histologic differentiation. In this study, similar retention or absence of ABH isoantigenicity was noted to occur in both primary gastric tumor and in its associated intestinalized epithelium. In addition to these studies, these antigens also could not be demonstrated[163] in over 80% of cases of carcinoma of the pancreas. With rare exceptions, the loss of antigen did parallel morphological anaplasia. Thus, the determination of alteration of blood-group substances may be useful in the early diagnosis and prognosis of malignancies of tissues that normally contain these isoantigens. Leukocytes from some leukemic patients also show alterations of the blood group antigen composition.[164] These alterations consisted of absent or decreased amounts of A and H antigens and presence of M and/or N antigens which are not detectable on normal leukocytes. All these antigenic alterations were significantly reversed by preincubation of the leukemic leukocytes with neuraminidase, suggesting that surface antigenic determinants in cells can be altered by glycoproteins sensitive to neuraminidase.

Loss of isoantigens controlled by the HL-A system has been demonstrated in a patient with lymphoma.[165] Most of the HL-A antigens on the lymphocyte specified by the patient's genotype could not be detected in cytotoxicity reactions with HL-A antiserum as the cellular population underwent dedifferentiation

to the neoplastic lymphoid cell type. That isoantigenic changes on peripheral lymphocytes occurred in association with malignant transformation was suggested by morphologic and biochemical changes in the circulating mononuclear cells which are characteristic of neoplastic lymphocytes.

A retrospective study[166] of the results of therapeutic castration for advanced carcinoma of the breast in 102 patients presents evidence for the possible association of blood types and the prognosis of cancer. It was found that patients with blood types B and AB had a higher objective response rate. The survival rates after initial therapy for these patients were as good as for those who were selected to have subsequent extensive ablative therapy after castration. Patients with blood type O had the lowest response rate and the poorest prognosis. It appears that blood group factors should be considered in investigations of therapeutic results and natural histories of cancer.

Elevated levels of serum ribonuclease have been observed[167] in patients with multiple myeloma and decrease in RNase levels was indicative of response to chemotherapy with the alkylating agents cyclophosphamide and melphalan. Enzyme levels observed in the urine suggest that high serum levels are due to excess entry of RNase into the serum rather than to a decreased capacity for urinary excretion of the enzyme.

Increased levels of ribonucleotide reductase, the enzyme catalyzing the conversion of ribonucleotides to deoxyribonucleotides, have been observed[168] in leukocytes from blood of patients with acute or chronic myelocytic leukemia. Much lower specific activities were demonstrated in lymphocytes from patients with chronic lymphocytic leukemia and even lower amounts in normal leukocytes. This increase may reflect the immaturity of the cell populations in AL and CML.

Medullary carcinoma of the thyroid is associated with an excess secretion of calcitonin. A sensitive radioimmunoassay[169] for calcitonin has been developed which can measure as low as 5 picogram per 1 ml of serum and may be used as a screening test for early diagnosis. With this assay it is possible to measure the serum calcitonin level of high risk patients, namely relatives of patients with the disease, in the absence of clinical signs and symptoms. Also the regression or progression of the disease after surgical removal of the tumor can be assessed by periodic determination of the calcitonin level.

Prolactin has been measured in unextracted human plasma by a sensitive and specific in vitro mammary gland culture system.[170] The results indicate that prolactin and growth hormone circulate as separate molecules in human plasma. The ectopic production of polypeptide hormones has been demonstrated for a variety of non-endocrine tumors. However, the ectopic production of prolactin by a neoplasm appears not to have been recognized. Markedly elevated serum prolactin levels have been observed[171] in two patients, one with undifferentiated bronchogenic carcinoma and the other with galactorrhea and hypernephroma. Therapy in both cases was followed by a decrease in the serum prolactin concentration. Thus, carcinoma should be considered in the differential diagnosis of patients with galactorrhea or hyperprolactinemia. In another study,[172] a single oral dose of L-dopa produced rapid lowering of prolactin levels in four

patients with chronically elevated plasma prolactin. Since L-dopa affects prolactin production, it may be of potential value in studying pituitary function and in reducing concentrations of the hormone in patients with breast cancer. Several experimental animal studies have associated prolactin with mammary tumorigenesis. In one study[173] it was shown that estrogen inhibition of established mammary tumor growth in rats could be overcome by administering prolactin. In another study[174] rabbit antibodies to rat prolactin when injected into tumor-bearing rats caused a significantly increased incidence of tumor regression and a significantly decreased growth of tumors. This supports the suggestion that DMBA-induced rat mammary carcinomas are prolactin-dependent.

A new rapid and sensitive radioimmunoassay[175] has been developed for serum human chorionic gonadotropin (HCG). This assay can be accomplished within a single work day. Further evaluation[176] has been made of the significance of the observed abnormality in serum human placental lactogen (HPL) activity in patients with molar pregnancy and trophoblastic tumor. The level of serum HPL activity in molar pregnancy is a reflection of the length of gestation and the histologic grading of the tumor rather than of the hormonal activity of the trophoblastic tissue as reflected in the HCG titer. The observation that the level of HPL is inversely proportional to the degree of malignancy may explain why some patients with molar pregnancy have been reported to have normal serum HPL activity and why choriocarcinoma may function like early normal trophoblastic tissue producing large amounts of HCG and little HPL, whereas the older, more mature trophoblastic cell does just the opposite. This observation may serve as a reliable index of the neoplastic potential of hydatidiform mole.

The etiology of neuroblastoma is unknown and the occurrence of this tumor in more than one member of a family has been rarely reported. The results of two studies, however, suggest that this malignancy may be inherited. In one family studied,[177] neuroblastoma was found in each of two siblings and in a parental cousin. In another study,[178] neuroblastoma was found in two siblings at 5 1/2 months of age. The father had increased levels of vanillylmandelic acid (VMA) in his urine, but there was no clinical evidence of a neural tumor. It is conceivable that neuroblastoma may be transmitted in an autosomal dominant pattern after mutation of the genes governing the neural crest formation.

The finding[179] of 2 infants with neuroblastoma localized to the region of the cervical dorsal nerve roots who had normal urinary catecholamine excretion has important implications in the diagnosis and follow-up care of these patients. These findings suggest that individuals whose tumor arises from spinal nerve roots and ganglia will have no abnormality in urinary catecholamine excretion. Consequently these analyses will not be helpful in distinguishing neuroblastomas from other spinal cord lesions and, more importantly, they will not be useful in evaluating the response to therapy of patients with primary epidural neuroblastoma. In contrast, neuroblastomas from the retroperitoneal area or from the posterior mediastinum which invade the spinal canal are associated with increased urinary excretion of catecholamines and metabolites. A simple, efficient spot test[180] for determining VMA has proved to be of value as a practical screening test in the initial workup of a child suspected of having neuroblastoma and is an efficient means of following the results of treatment. Data from an investigation[181] of urine and tissue cyclic

AMP activities in children with neuroblastoma suggest that increased catecholamine excretion and VMA in urine is not linked causally to raised amounts of cyclic AMP excretion or altered levels of leukocyte adenyl cyclase or diesterase.

A staging schema for neuroblastoma has been proposed[182] that is based on the extent of disease at diagnosis. The practicality of the staging has been illustrated by the ease with which 100 children with neuroblastoma entered in previous collaborative clinical research studies were staged according to the criteria advanced. In addition, prognostic implications could be derived from the staging. A special group, Stage IV-S, was devised for those patients who have localized primary tumors and remote spread confined to liver, skin, or bone marrow but who present with no involvement of the skeleton. These patients have a favorable prognosis and radiation therapy and chemotherapy may not be necessary in these children.

The elevation of bone marrow colony-stimulating factor (CSF) activity observed in patients with leukemia has been further evaluated. In a survey of sera[183] from 19 children with various types of acute leukemia, CSF levels were found to be elevated in all patients at some time during the course of their disease and elevations occurred with almost equal frequency in relapse and in remission. Elevations of CSF activity could not be correlated with chemotherapy or to the absolute numbers of blood granulocytes, lymphocytes, or monocytes or with appearance of granulopoiesis in bone marrow smears or the extent of bone marrow replacement by leukemic cells. Patients with acute granulocytic leukemia (AGL) have been characterized as often having a "CSF-loss syndrome", the patients losing excessive amounts of CSF in the urine and being less able than normal persons to develop high CSF levels in response to infections. Normal serum contains inhibitors which block CSF action while inhibitor levels were subnormal or undetectable in 60% of sera in patients with AGL. No correlation was observed between serum inhibitor levels and CSF levels in serum or urine, but there was a slight correlation between clinical status of the patient and serum inhibition levels.

Correlations appear to exist between serum copper levels (SCL) and cellular components of the bone marrow obtained on the same day from patients with acute leukemia.[184] The relationship between percentage of blast cells and serum copper is important since blast cells reflect disease activity. The correlation between SCL and percentage of blasts was most striking in the childhood cases but was also high in adult cases and the relationship can be expressed in terms of probability. Thus, the SCL is proposed as an important index of disease activity and of value for the management of acute leukemia. It has been suggested[185] that patients with acute leukemia have periodic determinations of serum precipitin and agglutinin antibody titers against Candida albicans from the day of admission. Signs of cutaneous and mucocutaneous candidiasis and saprophytic candidal reservoirs in the upper respiratory and lower urinary tracts should be sought. Routine institution of these procedures may facilitate not only early diagnosis and therapy but also understanding of the development of the serologic responses and reveal reasons for "false negative" precipitin reactions which now partially hamper successful serodiagnosis in

some patients. Also routine culturing of bone marrow at the time of hematologic examination would also be an effective method of monitoring the patient for mycotic infection.[186]

Recent chromosome staining techniques and the localization of satellite DNA have major implications for cytogenetics.[187] Each chromosome apparently has a characteristic staining pattern and slightly different distribution of satellite DNA, thus making each chromosome identifiable. It is also possible to identify added or missing segments. Pachytene chromosome mapping may eventually permit accurate delineation of the structural variability of each chromosome.[188] Provisional maps have been completed for chromosomes 21 and 22. The longer of the two consists of 10 chromomeres in a standard sequence and the shorter one of 6 such chromomeres, including in each case the large terminal chromomere within which the centromere is located. The two bivalents remain distinguishable by their having three and two major markers, respectively. Marrow chromosome studies[189] appear to have prognostic value in human "preleukemia" (myeloproliferative syndrome, idiopathic pancytopenia). If a marrow chromosome abnormality is detected in a non-irradiated "preleukemic" patient, the risk of developing clinical leukemia within the next few months is great, and patients who show such progression probably had subclinical leukemia already present in the marrow at the time of the study. If, however, frank leukemia does not appear within 3 months, "preleukemic" patients with marrow chromosome abnormalities are perhaps thereafter at no greater risk than comparable patients without such changes.

A continuing program to develop scanning agents for specific localization in tumors has resulted in the study of several compounds for pancreatic scanning. One of these thought to be of potential value is ^{125}I-1-(p-iodobenzenesulfonyl)-3-n-propylurea (^{125}I-iodopropamide). This compound is structurally similar to chlorpropamide and other sulfonylurea hypoglycemic agents whose primary site of action is on the beta cells of the pancreas. Tissue-distribution studies in the dog[190] showed that even though the total amount of the compound present in the pancreas was low, the concentration in islet cells as compared to adjacent acinar cells was high. Evaluation[191] of this compound in hamsters with a beta cell adenocarcinoma of the pancreas also showed a low pancreas-to-liver ratio. While it is unlikely that this compound could be a scanning agent for beta cell carcinoma of the pancreas in man, other labeled hypoglycemic compounds, particularly those that have been shown to have a necrotizing effect on beta cells will continue to be evaluated. ^{131}I-19-iodocholesterol has been used[192] successfully as an adrenal-specific photoscanning agent in a patient with Cushings syndrome. Since both adrenal glands appeared to concentrate the tracer equally and were similar in size, the clinical impression was confirmed that the patient had bilateral adrenocortical hyperplasia rather than a functioning unilateral adrenocortical tumor. The analog of chloroquine, ^{125}I-4,3-dimethylaminopropylamino-7-iodoquinoline (^{125}I-NM-113) has been used[193] in 28 patients as a reliable nonoptical, nonsurgical diagnostic aid specific for ocular melanomas. Melanotic melanomas showed a significantly higher than normal counting rate over the tumorous eye 14 days or more after administration of the tracer dose. Amelanotic melanomas concentrated the tracer equally well before 14 days but apparently lost the tracer at a faster rate. The diagnosis of ocular malignant melanoma can presently be made with a 95% confidence

when the mean difference in counting rates between the two eyes is 18% after 14 days. These increased counts obtained over the tumorous eye will aid in the specific diagnosis of ocular melanomas and prevent needless enucleation.

The barium radionuclides ^{135m}Ba and ^{131}Ba may be useful as bone-localizing tracers.[194] Both agents have significant advantages over ^{85}Sr (but also are partially excreted into the G.I. tract). Barium-135m has a half-life of 1.2 days and nonenergetic 268 keV emission. These properties make it nearly an ideal scanning agent despite the high internal conversion. Gamma camera and rectilinear scans can be obtained. Barium-131 has a half life of 11.6 days and has 3 principle gamma emissions which can be detected by rectilinear scanners. The two lower emissions are also detected by gamma-ray cameras. The economics of production suggest that both agents can be competitive with ^{85}Sr.

The accumulation of ^{85}Sr in soft tissue devoid of heterotopic bone and cartilage has been documented for the first time.[195] Autopsy tissue specimens of a patient with carcinoma of the oropharynx showed that the soft tissues infected with Aspergillus niger had 14 times the radioactivity of uninfected soft tissue. In vitro studies on the isolated fungus showed that these organisms strongly bind and concentrate ^{85}Sr. These findings may lead to the development of a radioisotopic diagnostic test for the in vivo detection of aspergillosis in humans. It is important to realize that the abnormal localization of ^{85}Sr noted on a bone scan performed in a cancer patient need not necessarily represent metastatic tumor.

Serial scintiphotographic studies[196] on 29 patients given bis-chloroethylnitrosourea and imidazole carboximide for various brain tumors suggest that scintiphotography may be of value in assessing the response of brain tumors to systemic chemotherapy. In this study the size and shape of focal areas of increased labeling proved more useful than did estimation of labeling intensity. The brain scan has been particularly useful in detection of intracranial extension of lymphoma.[197] Follow-up scans demonstrated improvement after effective radiation therapy and thus correlated with the clinical course.

An optical technique to perform automated platelet counts appears to have important advantages over electronic platelet counting.[198] Platelets and leukocytes from whole blood were enumerated by a darkfield microscope optical system that detects light diffracted by them. The method gave accurate and reproducible platelet counts, comparable with those of electronic particle counting of venous blood and substantially more reliable platelet counts on thrombocytopenic and finger-puncture blood samples. The superiority of the optical system over the electronic system appears to be due to the ability to count platelets in diluted whole blood rather than in plasma. This method should prove especially useful in performing the large numbers of platelet counts that are important for management of patients receiving chemotherapy.

PHARMACOLOGY

The search for naturally occurring tumor inhibitors continues in many parts of the world. Maytansine, a novel ansa macrolide has been isolated from

the fruits, roots and stem wood of Maytenus ovatus gathered in Ethiopia and Kenya. Datiscoside, an antileukemic curcurbitacin has been isolated from roots of Datisca glomerata gathered in California. Bleomycin, is a new cytotoxic antibiotic elaborated by a mutant strain of the soil bacterium, Streptomyces verticillus found in Japan during a search for antibiotics with an antitumor spectrum similar to that of phleomycin but lacking its nephrotoxicity.

Maytansine[199] is of interest partly because of its ansa macrolide structure, which includes carbinolamine, epoxide, or aryl halide functions, and thus is similar to the ansamycin antibiotics, rifamycins and streptovaricins which are antiviral and antimicrobial agents, and are of interest as inhibitors of RNA tumor virus reverse transcriptases. Maytansine also shows significant antileukemic activity in five standard animal systems over a 50 to 100-fold dosage range at the microgram per kilogram level, and cytotoxicity (ED_{50}) against KB cell cultures at 10^{-4} to 10^{-5} µg/ml.

Datiscoside[200] is of interest partly because of its significant in vivo activity against P-388 leukemia and Walker 256 intramuscular carcinosarcoma and cytotoxicity (ED_{50}=0.16 µg/ml) against human KB cells. Also, the structure of datiscoside, as determined by x-ray crystallographic analysis, establishes for the first time unambiguously the configurations of the curcurbitacins at C-20, assigned previously on biogenetic grounds, and at C-2, for which contradictory arguments have been presented in the literature.

Bleomycin (BLM)[201] is a complex of glycopeptides which can be fractionated and grouped into 13 sugar peptides with the main BLM-A_2 component present in at least 50% concentration. The exact mechanism by which BLM exerts its effect is still unknown, however, it markedly inhibits DNA synthesis, and to a lesser extent, protein and RNA synthesis. It has also been shown to produce DNA strand scission.[201] An in vitro study to elucidate the effects of bleomycin on cell survival and progression throughout the mitotic cycle of Chinese hamster ovary cells showed BLM to be most cytotoxic during mitosis. In order of increasing resistance were cells treated in G_2 phase, early S phase, late S phase, and G_1 phase. Interference with mitotic progression occurred only in cell populations treated with BLM in G_2 phase.[201]

In the course of clinical studies, the in vivo effects of bleomycin on the chromosomes of bone marrow cells were studied in four cancer patients, after treatment with BLM, (0.3 mg/kg i.v. or i.m. every 12 hours for 4 days, and the course repeated after an interval of 10 days). Chromosomal aberrations in increased numbers were observed in two of these patients and persisted for about one month after therapy. The aberrations consisted of hypochromatic regions, gaps, and breaks, all of which were of both the chromatid and isochromatid types. Histopathological studies of the marrow showed no abnormalities. The disparity between cytogenetic and histopathological findings remain unexplained. It is suggested that it reflects a dosage phenomenon similar to the results seen after low doses of radiation in which chromosome breakage can be seen in the absence of other significant bone marrow toxicity.

Seventy-five patients with various types of cancer were admitted to a clinical study of bleomycin.[203] An initial dose of BLM of 15 mg was followed by 30 mg two to three times a week administered either i.v. or i.m. Fourteen of 20 patients with epidermoid carcinoma of the head and neck region; 5 of 14 cases of lymphoma including Hodgkin's disease; 3 of 6 patients with testicular tumors; and one patient with lymphoangiosarcoma of the arm showed evidence of objective regression. Common side effects encountered were hyperthermic reactions, gastrointestinal disturbances, hyperkeratosis and vesiculation of fingers, alopecia and stomatitis. Pulmonary fibrosis is a rare but serious complication. There was no evidence of bone marrow, liver or renal toxicity. Blemoycin promises to be a useful therapeutic agent and merits further study.

A number of reports have described pharmacological and chemotherapeutic properties of various organic and inorganic platinum compounds. Those most active as inhibitors of cell division in E. coli and of development of several transplantable rodent tumors are cis isomers of Pt (II) and Pt (IV) existing as neutral complexes. The NH_3 groups of the totally inorganic species, or the NH_2 groups of the 5-membered organometallic ring established in the case of the ethylene diamine derivative, appear to be critical for biological activity, since substitution of NO_2 for NH_3 of the inorganic species has been found to yield a compound devoid of inhibitory action on bacterial cytokinesis.

In a further study,[204] cis-dichloro (dipyridine) platinum (II), (cis-PPC), an aryl congener of the totally inorganic antitumor compound cis-dichlorodiamine-platinum (II), was evaluated for pharmacological similarities to the inorganic complex. The parameters assessed were its effects on bacterial cytokinesis; development of the Ehrlich ascites carcinoma in vitro; on nucleic acid and protein synthesis in tumor cells in vitro; and on phytohemagglutinin-induced mitogenesis of human lymphocytes. E. Coli B was sensitive to the action of cis-PPC: virtually total inhibition of growth was achieved at an inhibition concentration of 8×10^{-5}M in a synthetic medium. Photomicrography showed that cells grown in medium containing 5×10^{-5}M cis-PPC were extremely elongated, quite analogous to the appearance of cells grown in the presence of the active inorganic platinum compounds. The survival times of mice bearing the Ehrlich ascites tumor were significantly extended; the greatest increase of mean survival time together with maximum 50-day survival was seen following a single 25 mg/kg dose which yielded 114 to 147% increased survival time with 2/10 50-day survivors. This aryl derivative possessed pharmacological actions similar to those of the inorganic platinum species but was somewhat less potent. The amenability of cis-PPC, having 10 potential tritium labeling sites, would facilitate studies of absorption, distribution, binding and excretion. Further binding and distribution studies using cis-PPC synthesized with tritiated pyridine are underway.[204]

Previous studies in normal and neoplastic tissues of mice indicated that the cellular toxicity of 6-thioguanine (6-TG) could be consistently correlated with the incorporation of 6-TG into DNA in place of the normal guanine component. A Phase I clinical study sought to determine whether the incorporation of 6-TG into the bone marrow DNA of humans is similarly related to dosage sequences; and to make a comparison of p.o. and i.v. dosages and the optimum

dosage for a 5-day course of daily treatments.[205] 6-TG was administered to 15 patients with acute leukemia, and to 9 patients with solid tumors. 6-TG and its metabolites were measured by labeling the 6-TG with radiosulfur.

Thirty-two pharmacological experiments were conducted with these 24 patients. In 7 patients with acute leukemia and in 7 patients with solid tumors, following single i.v. doses of 65 to 300 mg/sq. meter, the plasma half-times ranged from 25 to 240 minutes, with a median of 80 minutes. No correlation was observed between dose and half-time, although initial plasma levels of 6-TG did correlate with dose level. For the same patients, blood levels of 6-TG were higher after administration of i.v. dosages than after equal p.o. doses. Blood levels and the incorporation into bone marrow DNA were higher with the higher doses, but depended more upon the growth status of the marrow than upon dose level. Incorporation into DNA of the marrow was usually very small after administration of a single dose of 6-TG, but after 5 daily doses the guanine of the DNA was largely replaced by 6-TG, indicating that most cells were caused to enter DNA synthesis during the 5-day period.[205]

In a continuing search for immunosuppressive agents with minimal myelosuppressive activity, the effect of the glutamine antagonist Azotomycin on immune responses was studied by measuring the blastogenic responses of human lymphocytes to mitogens.[206] Doses of 1.0 µg/ml or greater completely inhibited responses to phytohemagglutinin, streptolysin O, and allogeneic leukocytes. Both tritiated thymidine incorporation and morphological manifestations of blastogenesis were inhibited. Inhibition could be achieved without toxicity, as demonstrated by stable viable cell counts and restoration of responses by washing the drug from the cultures. Specificity of action was proved by complete reversal of inhibition by L-glutamine. Addition of a wide range of L-asparaginase doses markedly increased the inhibition produced by Azotomycin. Certain dosage combinations were synergistic. The dose-limiting toxicity is not myelosuppressive for either L-asparaginase or Azotomycin but relates rather to gastrointestinal and central nervous system effects. For this reason, immunosuppressive therapy with these agents may not be associated with the increased susceptibility to infection which characterizes immunosuppressive doses of thiopurines, alkylating agents, and folic acid antagonists. This study, therefore suggests that, depending upon pharmacological factors, Azotomycin, either alone or in combination with L-asparaginase, may produce a potent immunosuppressive combination in man.[206]

A series of 2,4-diamino-quinazoline antifolates have recently been shown to inhibit L1210 leukemia in mice and dihydrofolate reductases from Streptococcus faecalis and leukemic cells. One of the more effective members of the series, methasquin, (NSC-122870) was selected for toxicologic studies in laboratory animals as a preliminary to clinical trials.[207] The treatment of laboratory animals with methasquin produces severe or fatal intoxication as a result of degenerative changes in the intestinal epithelium. These changes include severe weight loss, anorexia, emesis (dogs), diarrhea, and intestinal hemorrhage (dogs and monkeys). The high susceptibility of the intestinal mucosa to methasquin is similar to the sensitivity of this tissue to methotrexate (NSC-740) and aminopterin (NSC-739). Like the other 2,4-diaminopteridines, the quinazoline derivative can also induce depletion of hematopoietic elements in

bone marrow (rats and dogs) and megaloblastic changes in erythroid precursors (dogs). Methasquin has relatively low potency when given by the oral route compared with its activity by parenteral injection. Such findings indicate that the agent is poorly absorbed from the gastrointestinal tract. There are significant differences in potency of this quinazoline among different species. In rats and mice its activity is comparable to that of methotrexate. In dogs methasquin has potency equivalent to that of aminopterin. It is, therefore, suggested that the doses of methasquin to be used in initial trials in patients be guided by previous clinical experience with aminopterin.[207]

Adriamycin was isolated in 1968 from Streptomyces peucetius caesius. It is a hydroxymethyl analog of daunorubicin, with a higher therapeutic index than that of daunorubicin in several experimental tumor systems. Preliminary clinical trials with adriamycin gave significant responses in acute and chronic leukemia, lymphoma, Hodgkin's disease, neuroblastoma, Ewing's sarcoma, and in other solid tumors.[208]

In a later clinical trial adriamycin was given to 67 patients with a variety of tumors. The tolerated i.v. dose was 20 to 30 mg/sq meter/day for 3 days or 18/mg/sq meter/day for 5 days every 3 weeks. Objective responses were seen in 4 of 7 transitional cell carcinomas; in each of 2 liposarcomas; in 1 of 9 squamous cell carcinomas, and in the 1 adenocarcinoma of the breast. No responses were seen in 10 other soft tissue sarcomas; in 4 malignant melanomas, in 4 acute leukemias, in 1 lymphoepithelioma, or 5 tumors of the gastrointestinal organs, in 2 thyroid tumors, 4 kidney tumors, and 2 tumors of testis. At lo doses, nausea and vomiting, phlebitis, and alopecia appeared. At higher doses, mucositis and myelosuppression seemed related to the dose of each course, while EKG changes in 4 cases and 6 cases of congestive heart failure appear related to the cumulative dose. EKG changes, consisting of premature ventricular contractions and ST-T wave changes, appeared to be related to adriamycin in 3 cases. The congestive heart failures appeared related in 3 cases. Adriamycin appears to be promising in several tumors, but maintenance therapy may be precluded by cumulative toxicity.[209]

THERAPY

Permanent "cure", or at least prolonged continuous complete remission for children with acute lymphocytic leukemia (ALL) was the ultimate goal of a series of studies initiated in 1962. The rationale of these total therapy studies was based on extrapolation from animal studies, induction from clinical observation, and analogy between leukemia therapy and antimicrobial therapy.[210] Thus, a largely empirical sequence of proximate goals was constructed: 1. Attain remission efficiently and with minimal host toxicity; 2. Reduce the inapparent leukemia cell population with short-term high-dosage cytocidal therapy early in remission when the patient is better able to tolerate therapy and leukemia cell sensitivity is greatest; 3. Prevent or delay central nervous system (CNS) leukemia by destroying early in remission any clinically undetectable leukemia cells sequestered in CNS, and 4. Continue destruction of the subclinical leukemia cell population toward eradication with combination chemotherapy for two to three years.[211] The first four studies yielded the following data: An initial remission rate of 90% was attained using vin-

cristine and prednisone; 500 to 1200 rads of Cobalt-60 craniospinal irradiation early in remission did not prevent or delay CNS leukemia; combination chemotherapy was more effective in continuing remission when used at maximum tolerated dosage, and a 17% 5-year leukemia-free survival rate was attained. In the fifth study,[212] more intensive CNS therapy was given: 2400 rads cranial irradiation and twice weekly intrathecal methotrexate over 18 days during the first month of remission. Of 30 patients receiving all phases of therapy, CNS leukemia terminated remission in only 3 cases, and 19 patients remain in continuous complete remission for 2 1/2 to 3 years. If the remission pattern of the earlier studies persists in this later study, a 50% 5-year leukemia-free survival rate will be realized.

To define more precisely the value of radiotherapy alone in preventing CNS leukemia, and to assess the usefulness of the one-week course of intensive chemotherapy, the most recent controlled study was undertaken to test the following hypotheses: 1. A one-week intensive course of high-dosage, intravenous chemotherapy early in remission destroys sufficient additional leukemic cells to prolong continuous complete remission, and; 2. Irradiation of the craniospinal axis with 2400 rads early in remission destroys clinically undetectable leukemia cells which would not ordinarily be exposed to cytocidal concentration of chemotherapy and could later proliferate. Thus, CNS leukemia is prevented and complete remission is prolonged.

Of 110 children with ALL entered into this study, 104 (94%) attained complete remission. Of these, 94 children were available for the two randomizations. They first were randomly assigned to receive, or not receive, one week of high-dose intravenous chemotherapy (6-mercaptopurine, 1 g/sq m. daily X3; followed by methotrexate, 10 mg/sq m. daily X3, followed by cyclophosphamide, 600 mg/sq m. for 1 day only). Four weeks later, these patients were again randomized to receive, or not receive, 2400 rads Cobalt-60 craniospinal irradiation. Those patients randomized for no irradiation were to receive identical radiotherapy only if and when CNS leukemia developed. The one week of intensive chemotherapy had no effect on the duration of remission or on the frequency or the site of relapse, but irradiation had a marked effect. Complete remission was terminated by CNS leukemia in only 2 (4.5%) of 45 children who received this "prophylactic" craniospinal irradiation compared with 27 (55%) of 49 not so irradiated. Five of the 25 children who were given "therapeutic" irradiation for demonstrated CNS leukemia have already had recurrences despite continuous hematologic remissions. Under the conditions of this study it was concluded that: 1. One week of intensive chemotherapy does not prolong remission; 2. That 2400 rads craniospinal irradiation early in remission prevents or delays CNS leukemia and prolongs complete remission; 3. Once CNS leukemia develops, 2400 rads craniospinal irradiation is not sufficient to eradicate it; and 4. Any therapeutic regimen for childhood ALL must include CNS therapy early during complete remission if the goal is permanent cure. The results of past total therapy programs indicate that childhood ALL can no longer be considered an incurable disease.[212,213]

Two different regimens utilizing daunomycin, vincristine sulfate, and prednisone were administered in combination to treat 64 children with acute leukemia

in relapse. All of these patients had received previous therapy with vincristine, prednisone, and/or daunomycin but none had received these three drugs in combination. Seven patients were known to be resistant to both vincristine and prednisone. This triple combination was effective in inducing remission in 44 (69%) of these 64 children, but myelosuppression was severe and the results were not significantly better than the much less toxic combination of vincristine and prednisone.[214]

Eleven children ranging in age from 1 to 12 years with untreated acute lymphocytic leukemia at high risk for treatment failure were given a combination of prednisone, vincristine, daunomycin, and L-asparaginase for remission induction. Negro children and those with marked leukocytosis, hepatosplenomegaly, symptomatic visceral infiltration or other evidence of advanced disease qualified for this study. All 11 patients achieved complete marrow remission in a median time of 18 days. Reversible drug toxicity attributable to L-asparaginase included azotemia, hyperuricemia, hyperglycemia, hyperamylasemia, coagulation defects, and weight loss.[215]

A combination of intravenous cyclophosphamide and vincristine (Oncovin) and oral prednisone (COP) was given every 2 weeks to 262 patients with disseminated Hodgkin's disease, lymphosarcoma, reticulum cell sarcoma, and follicular lymphoma. A complete remission was produced in 36% of patients with Hodgkin's disease, 50% of patients with lymphosarcoma, and 39% of patients with reticulum cell sarcoma. These remission rates are significantly superior to those produced by single agents. Patients who achieved a complete remission were randomly allocated to monthly COP or to no treatment. The median duration of maintained remission was longer than unmaintained remission for Hodgkin's disease (42 weeks vs. 19 weeks) and lymphosarcoma (49 weeks vs. 21 weeks) but not for reticulum cell sarcoma (24 weeks vs. 25 weeks). The duration of remission for patients with little or no prior therapy was compared with that for patients in a similar study in which single agents were used. For lymphosarcoma and reticulum cell sarcoma, remissions maintained with COP were longer than remissions maintained with cyclophosphamide. For Hodgkin's disease and lymphosarcoma, unmaintained remissions after COP induction were longer than after single agent induction. All parameters of response, that is, complete remission rate, duration of remission, and survival were adversely affected by major prior treatment with radiotherapy and chemotherapy. The initial response to treatment correlated positively with survival. The survival of patients with lymphosarcoma or reticulum cell sarcoma receiving COP treatment was significantly superior to a comparable group of patients treated sequentially with single agents.[216]

A retrospective examination of the hematologic responses to total lymphoid radiation therapy in 100 patients with Hodgkin's disease (50 patients with, and 50 patients without prior splenectomy) indicates that the splenectomized patients seemed to have fewer and less severe episodes of thrombocytopenia, leukopenia, and related complications during their subsequent radiation therapy. In both groups, patients treated with a large combined para-aortic and pelvic field had a tendency for their blood counts to fall to their lowest levels at about 60 days, whereas in those patients treated with the three-field technique which includes the mantle, para-aortic and pelvic fields and

which is estimated to include about 75% of the active marrow as compared with only approximately one-half of a patient's active marrow for the two-field technique, the counts fell to nadir levels at around 85 to 90 days. This observation indicates that field size is more important that treatment time in determining the hematologic response. Moreover, the repopulation of the irradiated marrow might well be favored when there is no spleen available for the sequestration of migrating cells.[217]

Radiation therapy thus far has proved to be the single most effective therapy for lymphosarcoma and reticulum cell sarcoma. However, these neoplasms often present as generalized disease and limit the chance of achieving long-term survival through radiation therapy. Several encouraging reports in the literature that describe the cyclic use of combination chemotherapy led to a study to evaluate the usefulness of the cyclic administration of cyclophosphamide, vincristine, procarbazine and prednisone in combination for the treatment of advanced lymphosarcoma and reticulum cell sarcoma. Twelve patients with generalized lymphosarcoma and eleven patients with reticulum cell sarcoma were included in this study.[218] Therapy consisted of six 2-week cycles as follows: cyclophosphamide 600 mg/m^2 or 450 mg/m^2 i.v. on days 1 and 8; vincristine 1.4 mg/m^2 i.v. on days 1 and 8; procarbazine 100 mg/m^2/day orally for 10 days from day 1 through 10, and prednisone 40 mg/m^2 orally for 14 days from day 1 through 14, in cycles 1 and 4 only. No therapy was administered for 2 weeks between cycles. Therapy was stopped after 6 cycles. Seven patients with lymphosarcoma achieved a category 1 response, and of these, 4 are clinically free of disease. In the reticulum cell sarcoma group, only one patient achieved a category 1 response. Myelosuppression was severe in 4 patients (17%) and may have contributed to their deaths from infectious complications. A high incidence of extranodal involvement in this group of patients appeared to correlate with the low incidence of useful therapeutic responses.

Eighteen patients with advanced breast cancer, most of whom had received prior therapy with 5-fluorouracil (5-FU), hormonal agents, and several alkylating agents, were treated with a 5-drug combination (1 mg vincristine; 25 mg methotrexate; and 500 mg 5-FU, i.v. each once weekly. Cyclophosphamide, orally 100 mg/day after breakfast; and prednisone in divided doses, 45 mg/day the first 2 weeks, 30 mg/day the next 2 weeks, then 15 mg daily). In the absence of any early neurotoxicity, stomatitis, diarrhea, or bone marrow depression, these five drugs were continued. If reactions occurred, the drugs were titrated to the patients reactions. Seventeen of these patients had evaluable lesions. Eleven (65%) had objective regressions, defined as at least a 50% reduction in tumor size with no lesions showing progression. Most of the responders had had prior additive hormone therapy as well as 5-FU trials, neither of which prevented a later response to 5-drug therapy. The average duration of response was 6 months, and 3 patients continue in remission.[219]

In 1961 a protocol was drawn up for a randomized study comparing the results of treatment of advanced head and neck carcinoma with radiation only, versus the concomitant systemic 5-FU and radiation. A group of 55 randomized cases have been followed from a minimum of one year to a maximum of nine years. All patients selected were considered advanced cases with an estimated chance of cure less than 25%. Patients were staged using the TNM system. One group was

treated with external radiation alone, the second group was treated with external radiation plus concomitant systemic 5-FU. The improved cure rate and average survival of the entire second group was statistically significant; however the tabulated results indicated that the better results in the second group were present in the lesions originating in the oral cavity or oropharynx. All of these lesions were squamous cell carcinomas. The 106 lesions which were randomized in these two areas demonstrated an impressive advantage of the combination therapy. Reactions to the combination therapy are slightly more severe but within tolerable limits, and a greater number of long-term complications has not been demonstrated.[220]

The long-term survival and cure rates of one hundred and eight patients with advanced head and neck carcinomas were reviewed for the purpose of evaluating pre-irradiation intra-arterial infusion with 5-fluorouracil.[221] Half of these patients were treated with intra-arterial 5-fluorouracil infusion (3 to 15 mg/kg per day) prior to external irradiation (tumor dose of 6000-7000 rads over a period of 6 to 7 weeks, or 5000 rads to cervical areas where in a few cases preplanned neck dissections were done). The other 54 patients with similar advanced head and neck carcinomas, were irradiated without prior infusion. Twenty seven of 51 patients experienced tumor regression of 50% or more, but no increase in survival or cure rates was noted in the combination therapy group. Follow-up ranged from 3 to 9 years. Although there was a significant increase in the survivals of the patients who responded well to the intra-arterial infusion in comparison with those who did not, these investigators do not believe this is due solely to the intra-arterial infusion since the group as a whole did not demonstrate an increase in survival. It has been the experience of these and other investigators that a correlation exists between response to radiation therapy and response to chemotherapy.

Patients with cancer of the oropharynx, hypopharynx, and larynx were retrospectively staged according to the American Joint Committee for Cancer Staging and End Results Reporting categories.[222] Of the 68 patients treated with preoperative irradiation, 56 had low-dose radiation (2,000-2,500 rads, or less than 3,000 rads, given in 2 to 2 1/2 weeks followed in 1 to 14 days by surgery). Twelve patients had high-dose radiation (5,000 to 6,000 rads given in 6 to 8 weeks, followed by surgery within 15 days). Twenty-nine patients with carcinoma of the same areas and with a similar range of disease stages were treated by surgery alone.

There was no obvious difference in wound healing between the groups treated with preoperative irradiation regardless of dose when similar surgical procedures were compared. In all groups, wound healing became increasingly impaired with the extent of the surgical procedure, and less satisfactory as the size of the radiation field increased in the low-dose group. This was not noted in the high-dose group. Postoperative morbidity apparently increased with the extent of the procedure. Patients having surgery alone appear to have less postoperative morbidity compared with those receiving preoperative irradiation even with low doses. Morbidity increased with field size in the preoperative low dose group but not in the high-dose preoperative group. The local recurrence rate and survival at 2 years with and without disease appear to be less in both the low-dose and high-dose preoperatively

irradiated patients than in the surgery alone group. From a further analysis relating stage and site of disease to recurrence rate and two-year survival, it appears that the effectiveness of preoperative irradiation is most marked in lesions of the larynx and hypopharynx. A statistically significant decrease in local recurrence was noted in patients with clinically and pathologically positive lymph nodes in the preoperatively irradiated groups when compared with the surgery only group. The recurrence rate was 91% in the non-irradiated patients; it was 50% or less in the preoperatively irradiated patients, regardless of dose.[222]

A combination of hexamethylmelamine (NSC-13875), 6 mg/kg/day, given orally in four divided doses, and methotrexate, 12.5 to 50 mg/week, i.m., was given to 53 patients for lung carcinoma.[223] Twenty-five percent had a partial response, and 5% had complete remission. About 80% of these patients had transient hematologic and/or gastrointestinal toxicity. It is believed that a Phase III study will be necessary to determine whether there is any significant benefit of this combination over the use of each drug sequentially in the therapy of lung cancer.

Clinical studies of a combination of BCNU and Ara-C were conducted in 170 patients by the Southeastern Cancer Study Group.[224] Phase I studies were conducted in 17 patients with metastatic cancer, and Phase II studies in 153 patients with acute leukemia, predominantly myeloblastic. The majority of patients received 50 to 100 mg/m^2 BCNU i.v. followed by a 3-day infusion of 30 mg/m^2 of Ara-C. Only 1 of 17 patients with metastatic cancer responded to treatment. Of 153 patients with acute leukemia, 140 were evaluable, and 107 lived 21 days. There were 17 (9%) complete and 10 (6.5%) partial remissions. There are few clinical clues for determining the patient(s) likely to achieve remission; in this study the factors favoring remission were a higher initial platelet count, no prior therapy, female sex, and age greater than 60 years. Of all patients entered in this study, 30% died within the first 21 days. Analysis of the major cause of early death showed that 37% died of infection, 20% died of bleeding, 17% died of combined hemorrhage and infection, and the remainder died of unknown or unrelated causes.

Based on studies of the chemotherapy of human leukemia and late stage animal neoplasms which may be kinetically analogous to disseminated reticulum cell sarcoma, a therapy program was devised using sequential cyclophosphamide-vincristine followed by weekly methotrexate-cytosine arabinoside.[225] Each cycle of therapy consisted of cyclophosphamide 1.5 g/m^2 on day 0 and vincristine 1.4 mg/m^2 on days 1,8, and 15; subsequently 8 weekly doses of i.v. cytosine arabinoside 300 mg/m^2 and oral methotrexate 120 mg/m^2 over 24 hours were given followed by "Leucovorin rescue." Fifteen patients received 3 such cycles with intervening 2-week recovery periods; nine achieved complete remission and 6 had a partial response. Median remission duration was 10 months. Median survival from onset of disease was 15+ months and from beginning of therapy was 14+ months. Five of 7 patients who have relapsed are dead. Toxic manifestations included neutropenia below 1000/mm^3 in 10 patients; of these, there were recurrent episodes of sepsis in one. Alopecia occurred in 10, and reversible neurotoxicity in 8 patients.

The choice of treatment for patients with cerebral metastases from malignant melanoma has been influenced by the site and extent of involvement. Since most patients have multiple lesions, methods other than excision are usually necessary. To evaluate the response to whole-brain irradiation with chemotherapy and/or corticosteroids, the results of 41 patients with cerebral metastases from malignant melanoma were analyzed.[226] These patients received a median tumor dose of 3000 rads delivered to the entire brain over a two-week period. Twenty-four patients received concomitant chemotherapy and corticosteroids, and fourteen others received either chemotherapy or corticosteroids. The median and mean survival from completion of irradiation for all patients were 86.5 and 103 days, respectively. Sixteen patients (39%) showed definite neurologic improvement and had a median survival of 131 days. The median survival of the 25 nonresponding patients was only 17 days ($p<.002$). The median duration of improvement was 60 days (range 30-465 days). The most frequently seen responses included return of limb function; the disappearance of confusion, somnolence and coma, and cessation of headaches, nausea, and vomiting. Patients with neurologic improvement tended to have more indolent disease than those who did not respond.

Carcinoma of the ovary has become the fourth leading cause of cancer deaths among American women, following cancer of the breast, colon and lung. The palliative value of surgery and the tumor's responsiveness to radiation therapy, or to chemotherapy alone, suggest that a multiple therapeutic approach might achieve prolonged remissions or cure. A study[227] to assess the possible exploitation of certain biological characteristics of ovarian cancer by combining primary surgery with radiation and chemotherapy was done. Sixty patients with pelvic or abdominal dissemination received postoperative irradiation. Fifteen Stage II and 19 Stage III patients having more than half the tumor excised and no palpable disease postoperatively (Group A) had median survival times (MST) of 39 and 26 months, respectively. The MST of 26 Stage III patients with palpable masses (Group B) was 9.5 months. Thirteen Group A patients receiving radiation plus mustard therapy had a MST of 26 months. The mean free interval to recurrence was 18.5 months in the former category, and 13.5 months in the latter ($p=.02$). In Group B, similar response rates occurred with irradiation, or irradiation plus mustard therapy, but the latter regimen induced more complete responses and of greater duration with doubling of the MST. These findings suggest that survival time in advanced ovarian cancer is inversely proportional to the residual tumor volume after primary therapy, and reduction in tumor volume accomplished by surgery and irradiation is enhanced by chemotherapy. The basis for the progressive increase in survival observed with the addition of each therapy is believed to be therapeutically-induced stepwise decrements in numbers of surviving tumor cells. These conclusions are tentative, since they are based on a retrospective analysis of nonrandomized patients. However, the findings of this and other similar studies are encouraging and suggest the need for further evaluation in a cooperative controlled clinical trial.[227]

Many studies in animals and in human patients with leukemia are compatible with the notion that a further improvement in therapeutic outcome might be achieved by alternating intermittent use of chemotherapeutic agents having different mechanisms of action. Therefore a clinical trial was designed in

which adult patients with acute myeloblastic leukemia were treated with cyclophosphamide (CY), vincristine (VCR), and arabinosylcytosine (ara-C) given in a sequence designed to increase the proportion of surviving tumor cells which are proliferating, to synchronize their progression through the cell cycle, and to render them more vulnerable to an agent which interdicts DNA synthesis.[228] Courses were repeated at intervals determined by the recovery of normal bone marrow elements. Other patients were treated at similar intervals with short courses of cyclophosphamide given at high dose levels and the results compared. Thirty adults with acute myelocytic leukemia were randomly assigned to sequential treatment or to 4 doses of CY (25 mg/kg i.v.) given on successive days. The therapy consisted of a sequence of CY (15 mg/kg i.v.), VCR (0.025 mg/kg i.v. given 24 hours later, and a 48-hour i.v. infusion of ara-C begun 12 hours after VCR. Two courses of each therapy were repeated at 14-day intervals providing sufficient recovery of normal marrow elements. Both regimens induced remissions. The overall complete response rate was 50%. ^{3}H-thymidine and ^{3}H-arabinosylcytosine labeling indices of tumor cells were performed before treatment. High labeling indices for both compounds correlated with clinical responsiveness, but a high index for either compound alone was insufficient.

In 1968 a formal program was initiated for treating rhabdomyosarcoma.[229] Twenty children with rhabdomyosarcoma were treated in a coordinated program utilizing surgery, radiotherapy, and combination chemotherapy (vincristine, 1.5 mg/sq. m, cyclophosphamide, 300 mg/sq m, and dactinomycin, 0.4 mg/sq m, given i.v. at weekly intervals for 6 weeks, and then vincristine and cyclophosphamide were given every 2 weeks for 6 or 12 months). Therapy was determined by the stage of disease at which treatment was initiated. Of these 20 patients, 15 developed complete regression of tumor; 4 patients developed partial response. Of the 9 patients who are alive, 7 have been tumor free for 2 to 39 months. Concurrent radiotherapy and prolonged intensive combination chemotherapy are well tolerated following surgery for rhabdomyosarcoma. The coordinated use of these modalities offer prospects of curing more children with rhabdomyosarcoma.[229]

Immunotherapy

The varying clinical responses to therapy of similar appearing lymphomas, ranging from failure of regression, to remissions of many years duration, represent a challenge to the understanding of these diseases. One report describes the cases of two patients with disseminated malignant lymphoma that responded to limited chemotherapy and have remained free of disease for 11 and 8 years, respectively, after discontinuing chemotherapy. Both cases had initial clinical and histological features for a grave prognosis. In view of the accumulating evidence of the presence of malignant lymphoid cells in the circulation when the disease appears still localized, the hypothesis is made that the "cure" of lymphoma is not due to a destruction of all tumor cells, but rather to biological alteration in large numbers of lymphoma cells. It is inviting to think that in lymphomas, radiotherapy and chemotherapy introduce two beneficial effects concomitantly: first, they destroy large numbers of tumor cells, and second, they might tip the immunlogic balance in favor of the host. This might be achieved because the damaged cells maintain their

antigenic activity despite the loss of their reproductive capacity. Therefore, before most tumor cells are dead from radiotherapy or chemotherapy the immune mechanisms of the host have been stimulated by a larger number of antigenic tumor cells. When the number of tumor cells is decreased below a critical level, the immune mechanisms of the host are capable of containing or slowing down the proliferation of the remaining malignant cells. There is increasing evidence that the functional competence of the immune system of the host plays a considerable role in the development and behavior of tumors of lymphoid tissue.[230]

In view of the belief that at least some cancers in man may eventually be shown to have a viral etiology, it is reasonable to explore mechanisms by which the host normally counters viral infection. Recent experimentation has demonstrated that both the viral-inhibitory protein interferon, and chemicals capable of inducing interferon can prevent viral oncogenesis and produce tumor inhibition in animal tumor systems. At least one such interferon inducer, the synthetic polyribonucleotide Poly I:C, is now undergoing clinical trials in cancer patients at two Cancer Centers[231] in a dosage range from 2 µg/kg to 12.5 mg/kg. The drug is reproducibly pyrogenic in the majority of patients, but tolerance develops to pyrogenic effects of repeated daily injections of poly I:C. Poly I:C induced significant formation of interferon in about 70% of the patients studied to date. There was a strong, though not complete correlation between induction of interferon and the pyrogenic response. Interferon levels are generally detectable at 12 hours, maximal at 24, and clearly declining by 48 hours. Tolerance or resistance to further interferon induction develops and is maintained when the drug is given daily. Upon discontinuation, sensitivity is again evident within 7 days. The correlation between intravenous dosage of Poly I:C and interferon induction resembles an all or none response once a threshold of approximately 25 µg/kg has been exceeded. In contrast to the profound hepatotoxic effects in dogs, poly I:C has induced only modest liver function abnormalities in the clinical trials. No significant antitumor effect has been observed in man. An initial suggestion that the drug causes perturbation of clotting factors has not been borne out by extensive trials.[231]

Four different immunotherapeutic approaches were used during a 5-year study involving 55 patients with malignant melanomas:[232] immunization with irradiated autochthonous tumor cells; cross-transfusion subsequent to immunization by cross-transplantation of minced tumor; cross-transfusion subsequent to immunization by cross-transplantation of cultured cells; and innoculation of melanoma satellites with Bacillus Calmette-Guerin. Five patients had complete remission of their disease lasting from 3 months to 3 1/2 years; three of these patients presently are alive. Six of the patients showed partial responses. Antibody or cellular immune responses were demonstrated in five patients, three of whom showed concomitant clinical remission. The investigators suggest that immunotherapy will probably be most useful when the cancer cell mass is small. While it is too soon to suggest immunotherapy for primary disease, it does seem to be indicated for those patients who have had the major portion of the tumor mass removed at operation, or at the earliest sign of a recurrent metastatic lesion.

Intradermal injection of antigen probably represents the most effective route of administration, since adjuvant effects of skin components enhanced immunogenicity of the injected material and encourage the induction of the cellular immune responses. Useful adjuvant effects also might be obtained with Bacillus Calmette-Guerin, although its repeated administration or its use in tuberculin-positive persons could result in undesirable systemic reactions. Antibody against tumor cell antigens can, under certain conditions, enhance tumor growth. For this reason, care must be taken that this phenomenon does not occur in immunized patients. The cellular immune mechanisms play a major role in immunologic surveillance and target cell killing; consequently, the provision of competent lymphocytes to patients by either active or passive means appears to be of great import. Of interest, in regard to the administration of active lymphocytes, are the recent studies concerned with the injection of lymphocytes into patients with cancer after their in vitro exposure to phytohemagglutinin or cultured tumor cells. The potential value of established human lymphoid cell lines for this purpose is that such cell lines can be initiated readily from normal and diseased persons, and since they retain their tissue alloantigens in culture, large quantities of lymphoid cells of the desired histocompatibility types could be made available for immunotherapy. Tumor cells from the patients under study should be kept available in culture: immunization of patients with autochthonous tumor tissue or with cells from matched partners depends on the availability of proper tumor cell lines, as does response evaluation.[232]

Radiotherapy

Many modern megavoltage radiotherapy units are gantry-mounted to facilitate isocentric treatment methods which in turn facilitate dosimetry preplanning, since patient dimensions have only a small effect on the isodose contours near the rotational axis or isocenter. However, for multiple field and rotational therapy the depth of the tumor is critical, localization is three dimensional, and accurate beam direction is essential. A clinical facility and tumor localization procedure is described which promotes accurate and reproducible radiation therapy together with savings of equipment time and decreased extemporaneous demands upon the radiotherapist.[233]

The use of a special-purpose diagnostic roentgen-ray treatment simulator unit which duplicates the motions and positional limits of a 6 MeV Clinac-6 treatment accelerator, and provides a tumor localization roentgenogram is a useful part of the clinic. For tumor localization the patient is positioned with a specified, but provisional, reference point located at the isocenter of the simulator. This reference point is specified within the patient by 3 skin markings, and recorded on both anteroposterior and lateral orthogonal roentgenograms having a constant magnification factor, by marker crosses relative to standard anatomic structures and drawn on the patient contour drawing. A suitable treatment plan is devised and this determines the tumor point which is located coincident with the isocenter of the accelerator during treatment. The tumor point may differ from the original reference point. The anatomic coordinates of the tumor point are recorded and used to establish the final skin markings for positioning the patient for each therapeutic

session. The pre-set dosimetry parameters are calculated by the dosimetrist following the development of the treatment plan and are recorded on a Physical Report Form. Thus the treatment technician's only responsibility is for adjusting the patient's position, beam angulation and field size, attaching such accessories as wedge filters, and setting the precalculated values.[233]

In the early phases of radiation therapy special emphasis was placed on the rate of tumor regression. Radiotherapists now accept that large tumors are much more difficult to control than small tumors, and that radiotherapy alone can hardly be expected to produce dramatic results for a large tumor mass. The major emphasis is now on the permanence of local control or freedom from recurrence.

With this change in emphasis, a clinical study was undertaken to evaluate the efficacy of high dose, radical radiation therapy administered shortly after completion of an excisional biopsy, a simple excision, or when the recurrence was limited to tumor masses of only several centimeters in diameter.[234] A total of 42 patients with soft tissue sarcoma received a full course of radiation therapy. All treatment was with a Cobalt-60 source at 80 or 100 cm source-skin distance, with parallel opposed fields usually 25 to 30 cm long, but providing about 10-cm margins above and below the limits of the surgical scar. For the first five weeks of treatment the fields were generous, then during the sixth and seventh weeks the fields were reduced in steps so that the final week radiation was administered only to the site of probable residual tumor. More than 24 months following treatment, 20 patients are alive without evidence of local or distant disease. These results, when compared with the local recurrence frequency following radical surgery, suggest that this radical radiation therapy as an adjunct to surgery, combines to yield a higher rate of tumor control than may be realized by either therapeutic modality alone.

During the past ten years at one university, sixty percent of the 550 patients with breast cancer that have been referred for radiation therapy were for the treatment of metastatic sites. Although the sites of involvement and the treatment regimens used were too variable for meaningful statistical analysis, a review of the techniques employed revealed the evolution of a pattern of treatment which might be termed presumptive palliation. In this approach, it is advocated that individual sites of metastatic involvement be treated aggressively with the best equipment and therapeutic technology available with the goal of sterilizing the metastatic focus at the time of its earliest detection. By using this approach, troublesome or even catastrophic foci of disease may be eliminated permitting more effective palliation of the individual sites than may be accomplished by waiting until the metastases cause severe pain, loss of structural integrity, or severe malfunction.[235]

Cancer of the vocal cord is a highly curable disease with excellent salvage of radiation failures by surgery. In general, the histology is uniform, the disease progresses in predictable fashion, seldom metastasizes, and can be appraised with the laryngeal mirror as well as radiographically. Radiation therapy administered 4 times weekly with a mid-weekly as well as week-end rest is extremely well tolerated by normal tissues.

Patients given 6,075 rads of Co-60 irradiation in 27 fractions over 46 days for Stage I vocal cord lesions experienced no discomfort through the treatment course, no membrane formation was elicited within the larynx and the post-irradiation voice was good. Three year results with this regimen are 16 of 18 cases alive and free of recurrence. However, this time dose schedule to date has controlled only 3 of 5 Stage II ($T_2N_2M_0$) lesions available for 3-year analysis. Because of superior results in other reported series in Stage II $T_2N_2M_0$ lesions, present clinical practice is 6,500 to 7,000 rads at a weekly dose rate of 900 rads, treating 4 times weekly. A dose of 7,000 rads in 52 days treating 4 times a week is a ret dose of 1,988. Stage III lesions with supraglottic or subglottic extension, are radiocurable. A treatment scheme similar to that recommended for Stage II lesions should yield a 50% 5-year survival. The radiation failures should be subjected to laryngectomy and may still be salvageable.[236]

The present difficulties in the production of adequate beams for fast neutron therapy arise from the limited penetration of 14 MeV neutrons, and the large source to skin distances required, which require a total yield of 4×10^{12} neutrons/second produced by the deuterium-tritium (D-T) reaction. Presently available D-T generators have a source strength of only 1×10^{12} neutrons/second, and the metal targets used are inefficient. An all-tritium target, consisting of tritium gas, would be more efficient, but presents practical problems of compressing the tritium into a small enough volume to make a desirable source. These two primary problems may be solved by using a mixed deuterium-tritium beam and a bare copper target, cooled with liquid nitrogen, which becomes loaded with entrapped D and T as the beam starts up. Such a target conducts heat well and may extend the target life-time from about 200 hours to an indefinite life-time. The most probable explanation for the severe late effects seen in the lower abdominal areas of patients previously treated with fast neutron therapy is the relatively thick layer of subcutaneous fat which exists in these regions, and the fact that with fast neutrons at 14 MeV, absorbed dose to fat is 20% higher than absorbed dose to muscle. Blood vessels which lie in a layer of fat seem to be especially vulnerable and an excess of 20% in dose to these vessels could account for the severe fibrosis seen. It is to be noted that neutron beams of different energies may produce an absorbed dose in fat which differs from that produced in this series of patients. Consequently, each new beam must be studied individually.[237]

From preliminary research and present clinical experience it has become clear that clinical applications of fast neutron therapy should be limited to tumors of the head and neck. Present clinical experience with such T4 tumors is yielding a 2-year local control rate of about 40%. Chief among the radiobiological problems associated with the clinical use of fast neutron radiotherapy is the selection of the most effective dose-fractionation regimen. The radiobiological equivalent (neutrons /x-rays) at low doses is large and changes rapidly and probably differently in different tissues.[238]

It has been firmly established that hypoxia offers less protection against fast neutrons than against x- or gamma rays whether lethality is measured either by the decreased reproductive capacity of mammalian cells *in vitro* or by the death of the irradiated whole animal. The differences between the

Oxygen Enhancement Ration (OER) for fast neutron energy upon P-388 murine leukemia cells, and the lack of effect of neutron energy upon OER reported in human kidney-derived T-1 cells correlated well (perhaps fortuitously) with differences in responses of the same cells to irradiation with monoenergetic track-segments of beams of deuterons and of alpha-particles whose ionization densities (LET) are known. However, irradiation of the P-388 tumor cells with collimated beams of fast neutrons of different energies at 10 cm depth in a tissue equivalent phantom yields OER values lower than predicted by a consideration of average LET and is relatively independent of neutron energy. For irradiation from a planar array of neutron-emitting 252-Californium sources in a tissue-equivalent holder simulating a brachytherapy implant, the OER is disappointingly high (2.0), although still significantly lower than for irradiation with 226-radium gamma rays in the same geometry.[239]

Surgery

The experience with cystectomy in the treatment of vesical cancer has been improved in recent years with the use of an abdomino-perineal radical cystectomy allowing removal of the urethra in continuity with total removal of the bladder, perivesical fat and lymph nodes, prostate, and with the use of intraurethral formaldehyde. Intravesical instillation of formaldehyde for fixation of tumor cells has been found a useful precaution against implantation metastases. For all stages of disease, this technique appears equal to partial, simple, or radical abdominal cystectomy and clearly produces superior results with the deeply invasive tumors.[240]

Disseminated carcinoma of the breast may affect the nervous system in a number of ways. Early recognition of neurological symptoms caused by intracranial and intraspinal metastases will lead to rapid diagnosis, wherein lies the best hope of restoration of function by neurosurgical intervention. The judicious application of newer techniques for the relief of pain has improved the quality of survival for patients with advanced cancer. Pituitary ablation has a definite role in the sequential management of patients with disseminated cancer of the breast, and, because of its low morbidity and freedom from serious complication, stereotaxic cryohypophysectomy can be recommended for postmenopausal patients after initial trials of hormonal therapy.

The increasing demand and the expense of obtaining and dispensing fresh human blood components prompted a study of the actual quantities used during intensive combination therapy of childhood acute lymphocytic leukemia (ALL). All the platelet/leukocyte-rich plasma (PRP), fresh whole blood (FWB), and packed red blood cells (RBC) given to 128 children from 1962-70 were tabulated. The transfusions were subdivided by status of patient's disease, stage of therapy, therapeutic regimen, and primary indication. During the entire period of remission induction and combination chemotherapy and radiotherapy, the average child was given 3.2 units of PRP and 2.5 transfusions of FWB and RBC. In contrast, over 20 PRP units and over six FWB and RBC transfusions were given to the average child during the period of experimental chemotherapy after hematologic relapse and to children who were remission-induction failures. The quantities given during each stage of disease in sequential therapeutic regimens were remarkably similar despite the 8-year span of study. It was con-

cluded that children with ALL receiving intensive combination therapy require relatively small quantities of fresh blood components and that most components are given after relapse during experimental chemotherapy.[242]

Viruses have been used as an aid to cancer therapy in mice. The treatment of mice bearing ascites and solid tumors with bovine enterovirus-1 (BEV-1) resulted in regression of the tumors without any pathological effect on the host animals. Death of mice with lymphatic-leukemia L4946 was delayed after such treatment. The oncolytic specificity of the virus does not appear to involve the production of interferon, but requires specific adsorption of the virus to the tumor cells. The specificity of killing extends to cells in culture, since viral-transformed cells and oncogenic cells are susceptible to the virus, in contrast to cells of untransformed lines and cells of primary cultures, which are resistant. The basis of the specificity of BEV-1 appears to lie in the host cell membrane, i.e., adsorption specificity. It is unlikely that this specificity is unique, e.g., a specific receptor site, but rather it is probably a general interaction between tumor membrane and virus. It is the hope of these investigators that these observations may be extended to different human cancers and to in vivo therapeutic processes.[243]

Prognosis

To determine the relationship of chemotherapy-induced immunocompetence to prognosis, the in vitro blastogenic responses of the peripheral blood lymphocytes of forty patients with solid tumors, or with acute myeloid leukemia, were evaluated before and after intensive chemotherapy.[244,245] The responses to phytohemagglutinin (PHA) and to streptolysin-O (SLO) were correlated with tumor regression, clinical status, and length of survival after chemotherapy. A comparison of pre-treatment responses to PHA and SLO and the 24 to 48 hr post-treatment values showed a significant depression in all forty patients (PHA:$P<0.01$, SLO:$P<0.02$). In those 23 patients who showed tumor regression and improvement in clinical condition their in vitro blastogenic responses measured 8 to 10 days after the end of therapy not only showed recovery, but also a significant overshoot above the pre-treatment value. The survival of this group of 23 patients was significantly longer, (5 to over 13 months, mean 11.5 months) as compared with the group of 17 patients who experienced progressive tumor growth and poor survival (1 to 4 months, mean 2.1 months).[246]

The past quarter of a century has seen the introduction of a number of effective chemotherapeutic agents and the extension of radiation therapy for the management of Hodgkin's disease. To determine how these therapeutic developments have affected the survival of patients with Hodgkin's disease, a retrospective study was done,[247] comparing the survival of patients dying between 1950 and 1961 (Group 1) when only radiation and alkylating agents were available, with that of a second group, patients dying from 1964 onward, when vinblastine (1962) and procarbazine (1965) came into regular use at the Massachusetts General Hospital. In this study, those patients dying before 1950 were excluded because of the difficulty in obtaining information about them. Also excluded were those patients over the age of 52 whose known unfavorable clinical course could be expected to obscure an improvement in survival of the

entire population. A definite increase in median survival from 34 months, for the 1950-1961 group, to 56 months for the 1964 onward group was found. With due consideration for the pitfalls of any such retrospective study, it still seems improbable that this major increase found in longevity can be attributed to any artifact of the retrospective approach employed. It is believed that these findings reflect a true prolongation due principally to more effective therapy.[247]

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SUMMARY REPORT
OF THE
SCIENTIFIC DIRECTOR
GENERAL LABORATORIES AND CLINICS
NATIONAL CANCER INSTITUTE

July 1, 1971 to June 30, 1972

There are a number of different ways to describe the program of General Laboratories and Clinics. The first and most traditional manner is by the scientific and clinical disciplines. It is important to maintain this type of organizational structure because it is the same as that in the academic community and in national professional societies. Not only does it give our staff a necessary professional image, but more importantly, it fosters the interchange between peers of information and of criticism essential to good research.

Most of the disciplines of basic medical research are represented in General Laboratories and Clinics, namely Biology, Biochemistry, Physiology and Pathology. Trends in cancer research have led to emphasis of certain special areas by the establishment of two new laboratories, Cell Biology and Molecular Biology. The growing applicability of mathematical techniques to biological research has led to the recent formation of the Laboratory of Theoretical Biology which is just now beginning to be staffed. It is also of value to note the disciplines that are not included in General Laboratories and Clinics: microbiology (especially virology), toxicology and pharmacology. These are assigned to the NCI program areas of Etiology and Chemotherapy.

The clinical specialties are less broadly represented in General Laboratories and Clinics, since medicine is assigned to the Chemotherapy Area, and some specialties must be sought outside the Cancer Institute. Conversely several of our branches provide the only competence in their fields within the NIH Clinical Center. The Surgery Branch offers general surgery, and the Radiation Branch radiotherapy, for all of NIH. The Dermatology Branch is also the only resource of NIH in its field. In addition to care of their research patients, all the clinical branches operate sizeable clinical research laboratories and carry out very active research programs.

Medical research is applied research in the sense that its final goal is the maintenance of good health. An individual scientist, however, can only contribute to this goal within the area of his competence and interest. He alone can select his approach to the problem he has set, subject to the final judgement of his work by his peers. Nevertheless, the public, with good reasons, has claimed the right to set more specific goals for medical scientists. This was originally done by the establishment of the categorical research institutes within NIH, of which the National Cancer Institute was the first. More concrete guidance was provided in the form of liberal support for research toward the goals selected. The rapid growth of

support, especially in cancer research, requires us, in good faith with the public, to apply the resources given to us to approach, in the best way we know, the goals set for us.

This need has led to a categorization of the research effort of General Laboratories and Clinics in a manner different from that by research discipline. It is possible to group the research efforts of each laboratory and branch under four major headings. Three of these areas are general medical research categories: Cancer Cell Biology, Diagnosis and Therapy. The fourth area, Immunology, is one of special emphasis that cuts across the other three. Although each laboratory and branch is placed in a single area by a judgement of the major thrust of its research, most participate in more than one area. The laboratories and branches are listed below by the General Laboratories and Clinics research program areas:

Cancer Cell Biology	:	Biochemistry, Biology, Molecular Biology, Physiology and Theoretical Biology.
Immunology	:	Cell Biology, Immunology and Metabolism.
Diagnosis	:	Pathology.
Therapy	:	Surgery, Radiation and Dermatology.

The Cancer Institute has employed another method to coordinate research of the various disciplines in the approach to certain large problems. This is the establishment of task forces of both intramural and extramural scientists of various disciplines to lead research efforts against cancer of the major organ sites. The management of the Breast Cancer Task Force has been assigned to General Laboratories and Clinics. This Task Force has made a major planning effort to set goals for breast cancer research to identify and set priorities for needed research, and to implement its performance by the contract mechanism. With this background of experience a similar planning effort is in progress for the field of cancer immunology. This will serve both to coordinate such research within the organizational elements of General Laboratories and Clinics and of the other major program areas of the Cancer Institute, as well as to promote contract research under the priorities established by the plan. A third area of planning within General Laboratories and Clinics is in the detection and diagnosis of cancer. Although this plan is in early development, several major clinical research contracts have already been started under the supervision of the Office of the Scientific Director.

These three efforts account for almost all of General Laboratories and Clinics growing collaborative research effort. In the future we hope to work with the Chemotherapy program area of the Cancer Institute to develop a plan for cancer therapy that will coordinate our program area of therapy--surgery and radiation--more fully with the much larger effort in chemotherapy. Such a plan will allow the development of needed collaborative contract research in the surgical and radiation therapy of cancer, especially in combination with new drugs. A small beginning is also being made in planning in the more basic area of cancer cell biology. While the planning of basic

research is certainly difficult, and some say impossible, the participation of working scientists in the use of the convergence technique has been of great value in identifying intermediate goals and possible lines of research, particularly in suggesting specific projects for contract work.

CANCER CELL BIOLOGY

In the Laboratory of Biology, Drs. Gantt, Stromberg and Smith have continued their studies of aberrant methylation of nucleic acids. Methyl transferases of different types have been found in different tumor viruses, but the methyl acceptor is not in the virus, but in the host cell nucleic acid. These results support the hypothesis that aberrant nucleic acid methylation is a control event in malignant cell transformation.

The Tissue Culture Section is developing a homeostatic cell culture system for fine control of pO_2, pCO_2, pH, etc., with the potential of growing masses of cells either in suspension or on surfaces. This system will allow rapid accumulation of information on the nutritional and other requirements of cells in culture, with the long term goal of the definition of cultural requirements for the stable growth of normal or cancer cells.

Investigators of the Laboratory of Biochemistry are studying the composition and physiology of leucocytes, and have identified a serum factor that stimulates the incorporation of amino acids into guinea pig granulocytes. Elsewhere in this Laboratory work on distribution and metabolism of cytoplasmic DNA associated with the endoplasmic reticulum supports the idea that this DNA originates in the nucleus and serves as a messenger function. Studies also continue on the structure and biological significance of intracisternal A particles, virus-like bodies common in mouse tumors. The major protein has been isolated in amounts sufficient for structural analysis. RNA-dependent DNA polymerase activity has been found in A particle preparations, although with different properties than those of reverse transcriptase of known RNA tumor viruses. The research of the Tumor Pathophysiology Section involves the study of mechanism of tumor regression in hormone dependent mammary tumors of the rat. The lytic process on hormone withdrawal appears to begin in the tumor cells, with metabolic activity first in nuclear RNA, then in nuclear and cytoplasmic proteins, and finally in lysosomal enzymes.

A major effort in the Laboratory of Molecular Biology is the study of the regulation of gene expression, using two genes in E. coli for which controlled transcription had earlier been achieved in a cell free system containing cyclic AMP and its receptor protein (CRP). It has been found that in the presence of cyclic AMP, CRP binds to DNA. It seems likely that CRP alters DNA so that RNA polymerase can bind nearby to initiate transcription. Another major area of research involves the possible interrelations of the intracellular metabolism of cyclic AMP and the development of cancer. Since cyclic AMP is involved in a variety of intracellular processes, a single genetic event that interferred with its synthesis would have multiple effects. Earlier it was demonstrated that transformed fibroblasts could be made more normal with an analogue of cyclic AMP. Present studies are defining which which characteristics of fibroblasts are under cyclic AMP control, and how

transformation affects this compound's metabolism. It has been found that rapidly growing cells have a low level of cyclic AMP, but that the levels are high in contact-inhibited cells; perhaps cyclic AMP is the signal for growth arrest. Finally in collaboration with Dr. J. Boder of the Etiology Area, NCI, evidence has been obtained that expression of a gene in the transformation region of the Rous Sarcoma virus lowers the levels of cyclic AMP.

Research in the Laboratory of Physiology has included quantitative estimation of vesicle production and plasma membrane turnover in hormone secretion by the thyroid. Daily thyroglobulin synthesis amounts to more than half the dry mass of the cell, and would require one microvesicle per square micron every 6 seconds for transfer to the follicular lumen. Membrane required daily for this would amount to twice the dry mass of the cell! In other work it has been shown that both in intact reticulocytes and in cell free systems, globin synthesis cannot be initiated in the absence of hemin, because a translational repressor is formed. A project studying bone formation during chick embryogenesis shows that collagen is synthesized intracellularly on polysomes, is carried by vesicles to the cell surface for incorporation into the extracellular matrix of bone. Collagen synthesis parallels the synthesis of other cellular proteins in bone tissue cells, but not of collagen synthesis by cells of other tissues such as skin. Elsewhere studies have been made of the regulation of energy and of water exchange in rats with cachexia from growing tumors. Sodium depletion is not an important factor in the cachexia. Animals with growing but small tumors do not, as do normals, increase diet consumption with bulk dilution of the food. A method has been developed for the quantitative assessment of feeding behavior, independent of food intake; preliminary results with the method suggest that feeding is affected early in tumor growth.

IMMUNOLOGY

In the Laboratory of Cell Biology work continues to solubilize and purify both transplantation antigens and virus-specified tumor antigens. A specific soluble fraction of the H-2 transplantation antigen has been shown in mice to produce both immunologic tolerance and to enhance the growth of certain tumors. Similar fractions of a membrane bound tumor antigen from a lymphoid RNA virus neoplasma RBL-5 have been shown to produce resistance to transplantation of the tumor. Since the fraction has no infective virus it appears to contain a cellular, not a viral antigen. Other studies of cellular cytotoxic reactions, which have different specificities from those of antibodies, have detected certain new antigens in cells transformed by MSV, but which produce no infectious virus. Thus, these cell surface antigens also are distinct from virus envelope antigen.

In studies of glycolipid metabolism in viral tumorigenesis, viral transformation of mouse cells caused a reduction in the activity of N-acetyl-galactosaminyl transferase, an enzyme necessary for the synthesis of higher gangliosides. Investigations of the cross reactions between fetal antigens and tumor specific antigens in papova virus induced tumors has shown that fetal antigens are expressed in cells after malignant transformation, but they are different from tumor specific antigens.

In continuing studies of intracisternal A-particles, virus-like particles which are found in normal and neoplastic mouse tissues, particles isolated from several mouse myeloma lines and from neuroblastoma cells contain a major protein which is distinct from that of murine leukemia and mammary tumor viruses. Electron microscopy has demonstrated A-particles in mouse male and female gonadal tissue and in embryos, suggesting that vertical transmission occurs.

Protective immunity can be established in BALB/c mice against tumor induction by a small number of malignant plasma cells. Attempts are being made to produce similar immunity with tumor secreted immunoglobulins or their fractions. In another project the chemistry of the ligand groups of some 16 different mouse immunoglobulins with antigen binding activity is under study. The ligand groups include a variety of different polysaccharide moieties, phosphoryl choline, and nitrophenyl groups. Insoluble immunoabsorbants have been prepared to allow purification of some of these proteins in a single step. It has been possible to crystallize the combining site of two myeloma proteins with activity to phosphoryl choline. X-ray diffraction studies of structure are in progress. Such knowledge may eventually lead to in vitro synthesis of specific antibody molecules.

Patients with neoplastic disease have been shown to have delayed hypersensitivity skin reactions to extracts of tumor cells. In acute leukemia, reactivity is present in remission but negative in relapse. Patients with colon carcinoma react to a tumor antigen which is somewhat similar to, but distinguishable from the carcinoembryonic antigen of Gold. In melanoma two reactive antigens have been identified, of which one appears to correlate with the stage of the disease.

The application of sensitive radioimmunoassay methods has shown the alpho fetoprotein is not associated exclusively with hepatoma and embryonal cell carcinoma, but is elevated in many neoplasms and in liver disease, and is present in small amounts in normal adult persons. Serums from hepatoma patients in Uganda showed a remarkable stability in individual patients, which was not altered by ineffectual chemotherapy, but which did fall rapidly after surgical removal.

The research program of the Immunology Branch includes laboratory work in immunochemistry and in the biology of lymphoid cells, and research both in the laboratory and with patients on transplantation immunology and on the immunodiagnosis and immunotherapy of cancer. In immunochemistry, a major protein of the red cell membrane has been isolated and its component sequences nearly completely determined. These results provide the first structural evidence supporting the lipid bilayer model with intercalated protein containing a hydrophobic portion buried in the lipid bilayer and a hydrophilic portion exposed on the cell surface. Approaches are also being made to structural analysis of the histocompatibility antigens which give the recognition signals for cell to cell interaction. Carcinoembryonic antigen associated with cancer of the colon has been partially purified and amino acid sequence analysis undertaken, giving the first such information for any tumor associated antigen. In an attempt to develop in vitro assays for cell surface antigens a method, involving stimulation of protein synthesis of

lymphoid cells, has been devised, which can detect both major and minor histocompatibility antigens, as well as assays of cellular immunity in mice and guinea pigs. The biosynthesis of sialic acid is under study, since it may be a component of a normal clearance mechanism for cells with altered surfaces. Studies related to humoral immunity have provided a three dimensional model of an IgG molecule at 6A resolution. Chemical studies of amyloid, an abnormal protein found with some cancers and immune disorders, have shown two classes, one with the same amino acid sequence as the light polypeptide chains of immunoglobulins, the other with an unrelated sequence.

In the area of immunobiology of lymphoid cells, preliminary evidence suggests that in tumor bearing animals there is a block in the maturation of the lymphoid effector cell response, which should destroy target cells without humoral factor involvement. Evidence has appeared of another mechanism for target cell destruction, in which they seem to be sensitized for destruction by normal lymphocytes by coating with a new immunoglobulin, styled lymphocyte dependent antibody. Studies are in progress of the mechanisms that control the growth, differentiation and activation of lymphoid cells. It has been found that alterations in protein synthesis occur in lymphoid cells within minutes of their stimulation with mitogens or antigens.

Studies in transplantation immunology are related to marrow grafting in patients with aplastic anemia, which has been unsuccessful in our hands because of the development of graft-versus-host (GVH) disease. Yet, if it could be done successfully, it would have considerable value in cancer therapy, including immunotherapy; therefore the study of GVH disease has been undertaken in primates. In other projects the interrelations of histocompatibility antigens with susceptibility to various diseases is under investigation.

In immunodiagnosis, a radioimmunoassay for carcinoembryonic antigen has been developed for application in early diagnosis and in monitoring responses to therapy. A study in immunoprophylaxis has shown a small positive effect, using BCG to try to prevent induction of tumors in mice with methylcholanthrene. An animal study is in progress of the possible immunotherapeutic effect of "deblocking" serum. A beginning effort in immune therapy of human tumors will soon begin in collaboration with the Surgery Branch.

The program of the Metabolism Branch is directed toward two major goals; first, the definition of host factors that result in a high incidence of neoplasia, and second, the elucidation of the physiological and biochemical effects of a tumor on the metabolism of the host. In the area of host factors in neoplasia, studies have been made of the pathophysiology of immune defects in three major categories of immune deficiency associated with neoplasia.

The first category is the disease, ataxia telangiectasia, of which 25 patients have been studied, is an inherited disorder with cerebellar ataxia, oculocutaneous telangiectasia, frequent respiratory infections and a 10% incidence of cancer, has a defect in the development of effectors of the immune system. Antibody response to antigens is poor. Most patients lack IgA and IgE; IgA function is poor even when present. The thymus is absent or poorly developed, and cellular immune responses are poor. Studies of

thymectomized rabbits suggest that the thymic defect is related to that in IgA. The basic pathogenesis may result from failure of interaction of entodermal and mesodermal elements for normal tissue differentiation. This hypothesis is supported by the finding of high levels of alpha-fetoprotein in all 22 patients tested.

Twenty-five patients with intestinal lymphangiectasia have been studied. Their immune problem is short survival of effectors, due to loss of serum protein and lymphocytes into the gut through disordered lymphatic channels. Immunoglobulin levels are depressed, especially the slowly catabolized IgE, but antibodies can be produced on challenge. There is a profound lymphopenia, which appears to be especially marked in the long-lived thymic-dependent cells. This seems to be why these patients fail to make delayed immune responses or reject foreign skin grafts. Three of the patients have developed lymphomas.

In the third clinical problem studied, the Wiskott-Aldrich syndrome, which is a sex-linked recessive disease characterized by thrombocytopenia, eczema and frequent infections, there is a defect in antigen processing and recognition. Four of the 13 patients studied have developed reticulo-endothelial neoplasma. These patients make large amounts of immunoglobulins, but have no antibody response to many antigens. Delayed hypersensitivity responses cannot be elicited although the thymus is normal and lymphocytes transform normally in vitro in response to mitogens.

Studies of the pathophysiology of immune defects have included studies of another gastrointestinal problem, gluten sensitive enteropathy. With exposure to gliaden, these patients make large amounts of both IgA and IgM; most of this is antibody to gliaden. Jejunal biopsies from these patients grow in the direction of normal in vitro, but this improvement is inhibited by gliaden in the medium. In other areas of research it has been shown that 15-30% of normal man's bile pigment comes from sources other than the breakdown of red blood cells. The administration of glutethimide and phenobarbital markedly increases liver clearance of bilirubin in patients with Gilbert's syndrome. In acute intermittent porphyria the increased excretion of delta-aminolevulinic acid (ALA) is probably caused by reaction to a partial block in heme synthesis. The administration of hematin markedly reduced ALA synthesis.

With the rapid development of the field of immunology in recent years, the Director, NCI, recognized the need for a great increase in research in cancer immunology, especially in collaborative research, and in the fall of 1971 asked Dr. William D. Terry, Chief of the Immunology Branch, to coordinate the development of a national plan for expansion of this research. The first step in planning a draft of the research logic has now been completed. Successive elements of planning will include (1) the establishment of priorities within the research logic framework, (2) the identification of existing levels of research effort, both intramural and extramural, in each area of the plan, and finally (3) the estimation of resources necessary to support the additional research that will be required.

Some additional funds have already been made available. Approximately twenty new contracts are under consideration for funding before the end of the fiscal year. Two contract review groups are being established. One including both intramural and outside scientists will address the science of the proposals. The second group, intramural scientists from all programs areas of NCI, will give primary consideration to program relevance.

Present collaborative research is largely concentrated in the two general areas of tumor associated antigens, and in cellular tumor immunology. Major expansion is expected in these two areas, particularly the latter. Immunodiagnosis will also be given increased emphasis. It is expected that more and more immunologic tests for cancer will be proposed, and careful evaluation of each will be required. A bank of serum specimens from patients in all stages of cancer, and of controls will be established, to allow such an evaluation. Some contract work will also be undertaken in immunotherapy, and also in new technology for immunology support in tissue culture, cell fractionation and cell separation. Finally, there may be several large contracts for broad research efforts in centers of immunology research.

DIAGNOSIS

The Laboratory of Pathology provides anatomic pathology diagnostic support for all the clinical elements of the National Cancer Institute, and also for large parts of the clinical services of other institutes within the Clinical Center of the NIH. This work involved 248 autopsies and more than 6,000 surgical specimens. In cytopathology more than 5,000 specimens were reported, plus nearly 200 chromosomal analyses. This year a pathology data processing system was put into operation, which provides a computer index of all anatomic diagnoses. In addition the Laboratory of Pathology carries on its own research programs in the following fields: Immunopathology, Viral Oncology and Cell Biology, and Comparative Pathology and Carcinogenesis.

In immunopathology significant accomplishments include the induction of malignant lymphomas in mice under prolonged immunosuppression and persistent antigenic stimulation. These experiments provide model systems analogous to the induction of lymphomas in humans with organ transplants. Another finding is the ability of antilymphocyte serum and heterologous serum to reduce production of interferon, which may also relate to lymphoma induction in organ transplantation. An additional accomplishment has been the localization of IgE on the surface membrane of human basophils by electron microscopy.

In viral oncology and cell biology EB virus antibody titers were found to be elevated in patients with Hodgkin's disease of the lymphocyte depleted type. This became apparent only after the Hodgkin's disease series was subdivided into histological types. By the use of chromosome markers conclusive evidence was obtained of the epithelial origin of salivary gland tumors induced by polyoma virus. It was shown that the marmoset lymphoma cell line induced by Herpes virus saimiri was of chimeric nature, and therefore not of monoclonal origin. Evidence was obtained that cyclohexamide increases the activity of interferon inducers; this may point the way to the

production of human interferon for antiviral chemotherapy. Finally it was demonstrated that Rifampicin inhibits pox virus morphogenesis.

In the areas of comparative pathology and carcinogenesis it was found that the size and durability of asbestos fibers are key factors in the induction of mesotheliomas. The direct implantation of pellets of cigarette smoke condensate was found to induce epidermoid carcinoma in the lungs of rats.

Under the monitorship of the Office of the Scientific Director, General Laboratories and Clinics, a general program has been established for the diagnosis of cancer. At the present state of knowledge of cancer and its treatment, a major reduction could be made in cancer mortality by improvements in methods for cancer diagnosis and their application. Although there is some research in progress in diagnosis both intramurally and with grant support, the potential is so great that a major expansion is warranted. It is planned to develop this expansion through collaborative research contracts in order to achieve a fully coordinated effort. Several contracts are already in effect, two in the diagnosis of lung cancer, one in the diagnosis of cancer of the large bowel, and one in methods of cell separation that will be of value in cytologic diagnosis. A procedure has been set up for review of contract proposals at two levels, with provision for input from scientists outside the government. At the same time formal planning is in progress for this program of research in cancer diagnosis.

The diagnostic research plan has the goal of the diagnosis of tumor of the major organ sites; that is, those that encompass 75% of cancer (excluding cancer of skin) at a stage before metastases have occurred, when the lesion is surgically curable. With current therapy for solid tumors, the achievement of an 85% actuarial 10-year survival can be taken as evidence that the goal has been met. This will probably require the ability to detect consistently tumors no greater than 1 cm. in diameter.

The plan includes both general approaches to cancer diagnosis, and organ specific approaches. The general approaches include the possibilities of development of immunological or biochemical tests, or both, for cancer, whether site-specific or not. Better means are needed of identification of tumor cells in body fluids, and of means of sorting cells to enrich samples for diagnosis.

The approaches to organ specific diagnosis have been partly worked out for cancer of the uterus, lung and large bowel; cancer of the breast and prostate are to be added. There are four general areas considered under each specific organ, as follows: 1) development of procedures to assure both patient and physician participation; 2) the development of simple screening techniques applicable to large numbers of people; 3) the development of methods for exact localization of a small tumor in a patient found positive on screening examination; and 4) simplification of techniques, both for screening and localization, and the provision of all the resources necessary for full application. These four approaches are not equally applicable or necessary for each of the major organ sites. For example, in cancer of the cervix, a good screening procedure exists and localization is no problem.

What is needed is population and physician participation, and simplification of the technique.

The field of cancer diagnostic tests, both general and organ specific, deserves some additional comment. With the growth of interest in cancer immunology, two tests, those of Gold and of Tal, have shown some possibilities and we expect that many more will be developed in the future. Each of these tests must be examined very carefully and critically to determine its ultimate worth. The first promise of a new test, when full blown cancer is compared with normals, begins to fall apart when early, or treated, cancer is compared with other chronic diseases. If a test passes these more exacting trials, it must then be studied in cancer and non-cancer patients, carefully selected for age, sex, disease stage, and with more and more exhaustive history, clinical examination and other laboratory tests to assure the diagnosis. A difficult problem at this late stage of definitive evaluation is this: since such a test has at least tentative predictive value, a positive test in a "non-cancer" patient requires action. The lengths to which one will go to find cancer under these circumstances must be carefully thought out in advance.

CANCER THERAPY

The Surgery Branch continued the provision of general surgical support for the whole Clinical Center, as well as the performance of its own clinical and laboratory research. Operations were done on 680 patients (42% major procedures); 228 were the Surgery Branch's own patients, 273 were from other branches of NCI, and 179 were from other institutes. Surgical consultations from intramural sources totalled 804; there were also 704 requests from extramural sources.

Direct clinical research comprises four areas: advanced cancer of the cervix, cancer of the head and neck, sarcomas, and melanomas. In locally advanced cervical cancer, pelvic exenteration has given a 68% five-year survival; and in radiorecurrent disease, 39% five-year survival. In head and neck cancer, our craniofacial exenterative procedure for disease of the ethmoids have given a 60% survival. Preoperative radiation is valuable in decreasing morbidity, but not ultimate mortality. Chemotherapy preoperatively helps to reduce tumor size; long term postoperative use gives promise of increasing survival, and is under cooperative study. Another cooperative study, with NHLI, of taste and smell, showed that after removal of the olfactory epithelium, some function continued from accessory areas in the oropharynx. Laryngectomy also markedly reduced olfaction; the reason for this is under further study. With sarcomas, treated by amputation with or without drugs, the five year survival in osteogenic sarcoma is 12%, which chondrosarcomas 49%, and with those of soft tissue, 52%. From a finding in animals that anticoagulation can reduce metastases, we have started a clinical protocol for pre and postoperative anticoagulation; follow-up is too short for results, but operative morbidity is only slightly increased. In melanomas wide margin surgical excision prevents local recurrence in all but 7%, but distant metastases cause most of the five year mortality of 54%. In these and other solid tumors, a study is underway with the Laboratory of

Pathology on cytodiagnosis following fine needle aspiration biopsy of the tumor. In over 200 aspirations, false negatives have been only 5% and false positives zero. The technique has real promise, especially in breast lesions.

Physiologic studies have shown that in major cancer surgery there is sequestration of some 20 to 30% of plasma albumin for at least 24 hours after operation. The vigorous early use of replacement albumin in these patients has reduced abnormalities of physiological functions.

In research in tumor immunology it has been possible to demonstrate a common tumor antigen in all patients with sarcomas. Cell free extracts from human sarcomas were able to infect normal human embryonic cells in tissue culture. These transformed cells, but not uninfected cells, reacted with serum from sarcoma patients. Following the finding of a common tumor antigen in melanoma patients, a clinical trial of immunotherapy was begun, by BCG injection into tumor nodules in, now, 18 patients with advanced melanoma. Five showed some response. One had a complete remission of injected and uninjected nodules, that has now lasted 3 years. Two others had dramatic regression.

Serum alkaline ribonuclease levels have been measured in normals and cancer patients. High levels were found in patients with adeno and squamous carcinomas, but patients with melanomas and sarcomas were normal. Lymphocyte reactivity to phytohemagglutinin has also been studied in normals and cancer patients. It was found that with high preoperative activity correlates with a favorable prognosis after cancer surgery, although the reverse was not the case. In patients with sarcomas and squamous carcinomas, lymphocyte reactivity was impaired, or was suppressed by the patient's serum, or both. Skin contact sensitivity to dinitrochlorobenzene has been measured in normals and cancer patients, and found to be much reduced in the latter. The percent of cancer patients and normals showing grades of reactivity was: spontaneous flare, 41% vs. 96%; impaired reaction, 29% vs. 1%; and anergy, 30% vs. 3%.

Studies with methylcholanthrene induced tumors in mice have shown that an animal with a growing tumor is protected against tumor development when additional cells are inoculated intramuscularly, but when inoculated intravenously many more tumors develop, more even than in an animal with no pre-existing tumor. This facilitation seems to be due to a serum factor in the tumor bearing animal. Immunologic differences between a primary benzpyrene fibrosarcoma and its metastases have been shown in rats, for transplanted metastases grew uninhibited in animals immunized with the primary. This work supports the concept that metastases develop as a consequence of differential immunogenicity between the primary and the metastases. Studies in guinea pigs and in mice have shown that the intradermal inoculation of tumor cells can prevent growth of an intramuscular tumor challenge. Guinea pigs so protected have been found to have not only a cellular immune response, but a circulating tumor antibody of the IgM class, specific for the immunizing tumor. Finally, RNA has been extracted from lymph nodes or spleen of guinea pigs previously immunized by a mouse sarcoma. When this RNA was incubated with normal mouse spleen cells and injected into mice, the mice showed marked resistance to later challenge with live sarcoma cells.

The Radiation Branch has continued its nationally recognized research program in the therapy of Hodgkin's disease, Ewing's Sarcoma, and non-Hodkgin's lymphoma of the lymphocytic type. In Hodgkin's disease the recognition that early metastasis after conventional therapy means that metastasis was present early but unrecognized, and demands wide radiation, together with very careful clinical staging, has produced superb therapeutic results. In Hodgkin's disease of Stages I and II treated by wide portal radiotherapy alone, the five year survival is 90%. In Ewing's Sarcoma heavy radiation of the primary site together with intensive chemotherapy has improved the usual 10 to 20% two year survival to more than 60% in a large series of cases collected nationwide. In non-Hodgkin's lymphoma, the use of extensive, even total body radiation rather than palliative radiation, together with standard chemotherapy has given a five year survival of more than 50% in advanced disease. Plans are being made for a similar program of multisystem therapy in cancer of the lung.

Much of this work has been done with outpatients. Individual patient visits in the past year totalled 6,500, largely outpatients (inpatient beds total 10). More than 200 patients were seen in consultation, and were the source of a heavy service load. In addition small supporting research efforts in radiobiology and in radiation physics and instrumentation were maintained.

The Dermatology Branch studies the growth and differentiation of epithelium and lymphoreticular tissues in normal, hyperplastic, and neoplastic states, including both the basic biology of the skin and its clinical diseases One area of research is the development of therapy for cutaneous malignancies and hyperproliferative states. Topical BCNU has been used to treat basal cell epitheliomas. Of a series of 24, 11 disappeared completely and 10 others regressed in part. Results with CCNU have been much less impressive. CCNU was used with succes in psoriasis. A national cooperative group is being organized to test a series of new drugs in these diseases and in mycosis fungoides. Studies are in progress to elucidate the genetic defects in benign and precancerous genodermatoses, including Darier's disease, epidermolysis bullosa, porokeratosis, Ehler-Danlos sundrome, and xeroderma pigmentosum. In the latter, in which cancer frequently develops in areas exposed to the sun, there is a defect in DNA repair after exposure to UV light. Although lymphocytes, and from preliminary work, epidermal cells as well, take up tritiated thymidine very slowly after UV radiation, total DNA repair over a longer period may be normal, so the defect may not be the cause of the cancers that develop. Studies are in progress of virus transformation of these cells after radiation as another possible method of tumor productions. Another area of research is the study of the normal and diseased skin by electron microscopy, including scanning electron microscopy. Studies also continue of the pathogenesis of mycosis fungoides and of pemphigus vulgaris.

Work in progress on the immunologic role of the lymphocytes with emphasis on blastogenesis and cell division. Supporting work has included development of methods for obtaining lymphocytes or monocytes or both from peripheral blood. An autoclave has been designed and operated for tissue culture work that has a steam generator that can use distilled water.

Biochemical studies of skin include work on melanogenesis, collagen, biochemistry and on keratohyalin. This latter material, present in large amounts in the keratohyalin granules of epidermal cells, has been isolated from calve's hoof epidermis, purfied, and found to consist of a protein with large amounts of serine, histidine, arginine and glycine, and which is a polymer of 13 oligomers.

The Breast Cancer Task Force administers a contract program which has the goal of reducing the national toll of one of the commonest cancers by improved diagnosis, prognosis and treatment through clinical and experimental research. The Task Force, consisting of both intramural and non-governmental scientists meets annually for a detailed review of the program. Four sub-committees of NIH and university scientists which meet several times a year, are responsible for program development in Epidemiology, Experimental Biology, Diagnosis and Treatment. A Steering Committee of senior intramural personnel, monitoring scientific balance and program relevance, meets every two weeks, and the daily administration is performed by the Endocrine Evaluation Branch.

Early in the year the Task Force completed a linear array of the general research logic of an attack on breast cancer, which has served as the guide for the development and expansion of the Task Force program during the year. The present program consists of approximately 20 research contracts in five categories: discriminants (estrogen excretion patterns) for clinical prognosis, tumor models for the study of the biology of breast cancer, early diagnosis, preclinical drug therapy and immunotherapy. Twenty new contracts are now being established to begin implementation of the new research plan. These will emphasize therapy, especially combined therapy with both surgery and drugs; diagnosis, including work on thermography, mammography and immunodiagnosis; and experimental biology, emphasizing growth regulation in mammary cancer, including the effects of hormones and immunologic mechanisms, and biological events in metastasis.

SUMMARY STATEMENT
ANNUAL REPORT
July 1, 1971 through June 30, 1972

The LABORATORY OF BIOCHEMISTRY conducts research on biochemical events and control factors involved in normal cell differentiation, neoplastic transformation and tumor regression.

The PROTEIN CHEMISTRY SECTION (Dr. E. A. Peterson, Head) supports a continuing study of the process of leukocyte maturation. This program involves the collaborative efforts of Drs. W. H. Evans, S. R. Himmelhoch, and M. G. Mage, supported currently by Dr. T. Gessner (Research Associate), C. Grieshaber and F. Huang (Guest Workers). It is intended to provide information on the normal course of leukocyte differentiation as well as derangements of maturation encountered in various types of leukemia.

Quantitative cytological studies (by Dr. Evans and Mrs. S. Wilson) of the proliferation and maturation of neutrophil granulocytes from the marrow of the guinea pig in short-term suspension cultures have revealed an inverse correlation between the absolute number of mature neutrophils in the initial suspension and the increase in the number of these cells after 24 hours. This increase was 6-fold in cultures from animals whose marrow had been depleted of mature neutrophils by an inflammatory stimulus in vivo. No significant decrease in immature neutrophils was noted during the culture period, indicating that cell proliferation occurred as well as maturation. The ability of normal guinea pig serum to enhance the incorporation of labeled amino acids specifically into protein of granulocytes, preliminary indications of which were reported last year, has been substantiated by further work (by Drs. Grieshaber, Evans and Himmelhoch), including the significantly greater effect of serum from leukopheresed guinea pigs. The serum factor responsible for these effects does not pass through a cellophane membrane, and ion-exchange chromatography has shown it to be associated with only a small fraction of the serum protein. Investigations by Drs. Evans and Huang on the incorporation of tritiated glucosamine into the TCA-insoluble fraction of granulocytes separated from other bone marrow cells by velocity sedimentation at unit gravity have shown that mature granulocytes (polymorphonuclear cells and band cells) incorporate glucosamine at a much greater rate than immature cells. These results confirm those obtained by autoradiography of labeled cells last year.

Drs. Himmelhoch and Gessner have shown that leukocyte myeloperoxidase is a TPNH-oxidizing enzyme, and obtained preliminary evidence indicating that it may be the only such enzyme in the guinea pig leukocyte. Since TPNH generation is a central requirement for phagocytosis, it appears that myeloperoxidase may be involved in the biochemical basis of that important function of the leukocyte. Drs. Gessner, Himmelhoch, and Shelton have found that the "crystalloid inclusion" of the eosinophil granules could be liberated in intact form by careful homogenization of the cells in the presence of critical amounts of a nonionic detergent. It is tentatively assumed to represent the major protein component seen in gel electrophoresis of extracts of these

granules. A method for isolating eosinophil granules uncontaminated by other subcellular fractions has recently been developed and will be used in further investigations of the granule components.

As a result of several years of experimentation, Dr. W. O. McBride has made the important observation that mouse L cells could be biologically transformed by incubation with isolated metaphase chromosomes of the Chinese hamster. The transformed cells were detected at a low frequency (about 10^{-6}) by growth in selective media. This frequency appears to be greater than the reversion rate of recipient cells in control cultures. Physical characterization of the gene product (HGPRTase) from three unequivocally separate colonies isolated in one experiment employing CH (wild type) donor chromosomes and mutant (HGPRTase-lacking) recipient mouse L cells has excluded the possibility that these colonies arose as a result of reversion. The HGPRTase from each of these transformant clones is indistinguishable chromatographically and electrophoretically from the enzyme obtained from CH cells, whereas it clearly differs from mouse L cell HGPRTase by both of these criteria. A rare revertant isolated from control experiments contained HGPRTase identical to the wild-type mouse enzyme, as expected. Although the three transformed clones all produce the same HGPRTase gene product specified by the ingested chromosomes, they differ in karyography and in the stability of the phenotype in nonselective media.

Within the NUCLEIC ACIDS SECTION, Dr. W. C. Schneider (Head) has continued his studies on the DNA associated with the endoplasmic reticulum (microsomes). This cytoplasmic DNA has now been detected in spleen, kidney, brain, pancreas, submaxillary gland, and a plasma cell tumor and could be differentiated in most cases from nuclear DNA on the basis of the amount of thymidine incorporated. Hybridization studies indicate that microsomal DNA is closely related to nuclear DNA and support the idea that this DNA originates in the nucleus and serves a messenger function.

Dr. Anderson, with Dr. Garvey and Mr. Liacouras, has continued studies on two key enzymes in the synthesis of RNA: uridylate kinase and uridine kinase. The mechanism of the uridylate kinase has been studied in detail with a 300-fold purified enzyme from Tetrahymena and it has been found that the enzyme apparently forms a ternary complex with both substrates rather than with one substrate at a time. These studies are important in determining how this activity, and hence RNA synthesis, is controlled. The uridine kinase has been purified about 200-times from a drug-sensitive tumor in preparation for similar studies on its mechanism. Dr. Rotherham has begun studies on nuclear fractions obtained from mammary tumors that can be induced to regress (see below) with the objective of determining whether changes in these fractions can be detected and correlated with regression. Dr. Otani has synthesized a number of β-hydroxy amino acids related to methionine. These compounds will be tested for potential inhibition of tumor growth.

Dr. W. Kidwell has continued studies dealing with regulation of the cell cycle, particularily with regard to factors that may control initiation of DNA synthesis. Utilizing an in vitro DNA synthesizing system (isolated L-cell

nuclei) described previously, cytoplasmic factors were detected which differentially stimulate the labelling of certain categories of DNA (satellite). These factors were found only during S-phase of the cell cycle. They have been separated chromatographically into two fractions and further characterization is proceding. In collaborative studies with Dr. H. Ozer, Dr. Kidwell has also found that SV40 virus contains a specific endonuclease which can cause single strand breaks in the closed circular viral DNA and may play a role in viral replication and cell transformation. He and Dr. Scott (Laboratory of Experimental Pathology, NIAMD) have also found that 70 Å particles present on the cell membrane and apparently involved in the binding of viruses undergo dramatic changes in number during the cell cycle, and changes in arrangement during contact inhibition.

Studies were continued in the BIOSYNTHESIS SECTION on the structure and biological significance of intracisternal A-particles, virus-like structures that occur with low frequency in adult and embryonic mouse tissues but are common in many transplanted and primary mouse tumors. Collaborators have included Dr. E. Kuff, Mrs. K. Lueders, Drs. D. Marciani (Guest Worker), H. Ozer, S. Wilson, and N. Wivel (Laboratory of Cell Biology, NCI). During the past year, specific A-particle antigen was detected in several additional tumor sources and in normal mouse embryo factions. Dr. Marciani has isolated the major A-particle protein from three separate tumor sources in amounts sufficient to permit amino acid analysis and peptide mapping.

Of interest was Dr. Wilson's observation of RNA-dependent DNA polymerase activity in A-particle preparations from several different tumors. The enzyme specifically promotes synthesis of poly(dT) in the presence of natural or synthetic RNA templates that contain poly(rA) sequences. This restricted range of template specificity and certain other properties distinguish the activity from the reverse transcriptase associated with known RNA tumor viruses. With oligo(dT) as primer, the enzyme was active with messenger RNA prepared from rabbit reticulocytes and also with RNA isolated from the A-particles themselves.

Intracisternal A-particles may represent the expression of an integrated viral genome distinct from those of the C- or B-type particles. In collaboration with Dr. J. Minna (Laboratory of Molecular Genetics, NHLI), the regulation of A-particle expression is being studied in hybrid lines resulting from fusion of mouse cells containing widely different amounts of specific antigen, and in interspecies hybrids (mouse:man and mouse:rat) where one parent line is devoid of antigen.

Other aspects of the control of specific gene expression are under continued investigation by Drs. E. B. Thompson, J. Miller (Research Associate) and D. Aviv (Guest Worker), who are using as their main experimental model the steroid-inducible enzyme tyrosine aminotransferase (TAT) in hepatoma tissue culture (HTC) cells. Hybrids resulting from fusion of TAT-positive HTC cells with those of various TAT-negative lines (e.g. 3T3 fibroblasts, L cells, BRL62 hepatocytes) have all failed to express TAT. This repression can occur very shortly after cell fusion, reducing the likehood that is due to

selective chromosome loss. Regulation of TAT-synthesis at the translational level has been postulated to explain these and other effects; however no direct evidence for this type of control was obtained with a cell free amino acid incorporating system enriched for ribosomes specifically engaged in TAT synthesis.

A by-product of the above studies was the observation that hybrids derived from fusion of L cells and HTC cells showed contact inhibition of growth, whereas neither parent did so. Each of the transformed parent lines is known to produce C-type virus particles; particle production continued at a high level in the contact-inhibited hybrid. Additional cell fusion studies on the regulation of contact inhibition are in progress.

In collaboration with Drs. M. Lippman (Research Associate) and S. Perry of Chemotherapy, NCI, steroid binding techniques devised for study of HTC cells have been extended to human and mouse leukemia cells. The concentration of specific steroid binding sites varies among different types of leukemic cells. Steroid binding capacity is being correlated with response to steroid therapy in an effort to provide improved discrimination in the clinical treatment of leukemia.

Dr. E. Shelton and W. Bennett (Research Associate) have progressed in developing an experimental system in which to examine in detail the interaction of lymphocytes and macrophages cooperating in the immune response. Peritoneal cells from mice pre-immunized with either sheep erythrocytes or horseradish peroxidase (HRP) could produce strong secondary responses to the antigens while being maintained in diffusion chambers implanted in new hosts. Cells producing anti-HRP antibody could be visualized by histochemical reaction of bound HRP. A ferritin-labelled antibody procedure is being developed for electron-microscopic detection of antigen (HRP) bound to cell surfaces.

Also during the past year, Dr. Shelton has succeeding in elucidating by electron microscopy the complex configuration of complement component Clq. This very labile molecule has a remarkable structure consisting of 6 flexible strands radiating from a core area and each carrying a terminal subunit. The data provided by this study will be of basic importance in understanding the interaction of Clq with antigen-antibody complexes.

The activity of the TUMOR PHYSIOPATHOLOGY SECTION (Dr. P. M. Gullino, Head) is concentrated on the study of the mechanism of mammary tumor regression. Hormone dependent tumors of the rat are used as models. Participants in this study are Drs. Gullino, Y. S. Cho-Chung, R. Lanzerotti (Research Associate) and R. Knazek (Research Associate). Experiments completed thus far indicate that tumor regression is mostly due to an autophagic process which appears to destroy cells at random.

Lack of blood supply, inefficient utilization of blood born metabolites and invasion by immunological type of cells do not appear to play a decisive role in tissue lysis. Tumor regression initiates a few hours after hormone deprivation and is characterized first by a sharp increase in substrate incorporation into nuclear RNA; augmented incorporation of substrates into protein and lipids of nuclear and cytoplasmic fractions follows. A few hours later, increased activities of several lysosomal enzymes are detectable. The increase in activities of acid ribonuclease and β-glucuronidase appears to be

due to synthesis of new protein molecules which are localized within the regressing cells rather than in the interstitial spaces. It is not clear whether these events are instrumental in initiating regression or are the consequence of tissue lysis.

Since tumor regression is a process which involves an organoid structure of the living host, the experimental approaches have thus far used tumors growing in animals. Attempts are being made to grow neoplastic mammary cells in artificial capillary nets until they form organoid structures in vitro. The results thus far demonstrate that neoplastic cells can be grown in artificial capillary nets to form cell aggregates. However an in vitro organoid structure of the tumor has not yet been obtained. Work in this area is progressing with the objective of developing an in vitro model of tumor regression.

Within the CYTOCHEMISTRY SECTION, Dr. D. Burk (Head) has been studying the gas adsorptive properties of charcoal preparations with regard to their relative efficiency as cigarette filters. Large and readily measured quantities of gas were promptly eluted from various charcoals and charcoal filters upon simple addition of water or other liquids; and the greater amount of adsorbed gas as measured by this index, the greater the effectiveness of a given charcoal in removing acutely cell toxic cigarette smoke components. Charcoals placed as filters in cigarettes are known to show marked decrease in adsorptive capacity over periods of days to months. The present study indicates that a major factor in this reduction of capacity is the distillation of water from the tobacco to the charcoal filter. A significant recovery in the ability of the charcoal to remove toxic smoke components was achieved by brief heating of the whole cigarette or the filter alone.

Dr. M. Woods has followed the glycolytic properties of a group of transplanted mouse liver tumors through consecutive transplants. The glycolytic patterns remained remarkably constant during a year or more of transplantation, and rather strongly resembled patterns encountered in primary hepatomas in the same mouse strain. A large number of the primary tumors were studied and in most cases compared with adjacent tumor-free portions of liver. In some 100 primary liver tumors studied to date the glucolytic capacities of the tumors have been very significantly in excess of those occurring in the host livers or in livers of tumor-free mice. Very limited apparent exceptions were found last year in certain large but non-necrotic primary hepatomas in 21 month old C_3HfB mice. An attempt to recreate these peculiar "normalized" tumors is underway. The overwhelming amount of data obtained so far in these investigations support the view that the origin and progression of malignancy always involves an increased capacity to carry out glucolysis anaerobically, especially an increased capacity to utilize low levels of glucose and a decreased susceptibility to anti-insulin (steroid) regulation. There is considerable evidence that glucolytic capacity plays a crucial role in the functioning of defensive cells of the RES. Successful chemotherapy may depend upon knowledgeable exploitation of differences in glucolytic regulation in cancer and the normal defensive cells (RES).

Serial No. NCI-375
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Homogeneity and structure of proteins

Previous Serial No.: Same

Principal Investigator: Elbert A. Peterson

Other Investigators: M. Clyde Otey

Cooperating Units: None

Man Years:
Total: 2-1/2
Professional: 1-1/2
Other: 1

Project Description:

Objectives: To develop methods and procedures for the separation and analysis of proteins and to apply these methods to the purification of specific proteins and the study of their structure-function relationships.

Methods Employed: A variety of polyanions and polycations differing in molecular size, charge density, and chemical composition are prepared by appropriate reactions. Most of these are ionic derivatives of low molecular weight dextrans, but other types are being explored. They are evaluated as displacing agents, using test protein mixtures, on cellulosic ion exchangers. Effluent fractions are evaluated by electrophoresis in slabs of polyacrylamide gel, using procedures described in the report for 1969-70.

Major Findings: The initial phases of the development of a displacement method for the ion-exchange chromatography of proteins and other macromolecular polyelectrolytes were reported last year. Further development has been complicated by the unexpectedly high concentration of counter-ions released from the adsorbent as the concentration and the charge density of the displacing molecules were increased to achieve sharp displacement of proteins having very high affinity for DEAE-cellulose. Apparently, the small size of the displacers (M. W. = 15,000 to 20,000) and the high content

of carboxylate groups required to confer high affinity on such small molecules resulted in a degree of efficiency in interaction with the charged sites on the adsorbent that vastly exceeded that observed in the past with proteins, nucleic acids, etc. In some experiments more than half of the counter-ion initially bound by the adsorbent emerged at concentrations approaching 0.1 M, wiping out large portions of the displacement train because of its interference with adsorption.

The concentration of salt released by displacement of the counter-ions could be brought to an acceptable level by a number of devices, including the use of low concentrations of displacer, dilution of the adsorbent with inert material, and the use of displacer of higher molecular weight and lower charge density. Each of these approaches has significant disadvantages. Recently, however, very promising results have been obtained by applying the final displacer in the free acid form to a column previously charged with the spacer mixture of low affinity CM-dextrans in the salt form. Under these conditions no counter-ions are released from the adsorbent by the final displacer, and those released by the spacer mixture emerge ahead of the displacement train and do not interfere.

A general procedure has been devised for the preparation of mixtures of CM-dextrans, each mixture representing a continuum of molecules of successively increasing carboxylate content and, hence, increasing affinity. Simple modification of the procedure permits alteration of the distribution of the affinities over a wide range. These mixtures serve to space the proteins in the displacement train.

The preparation of suitable polycationic displacers has proven, thus far, to be much more difficult than the preparation of polyanionic ones.

Significance to Cancer Research: Displacement chromatography of proteins, nucleic acids, etc. promises to be of considerable value at any scale of operation and therefore has significance to all research that involves the isolation of such substances. Its immediate significance to the program of this Section, however, lies in its potential application to the fractionation of the small quantities of protein available in each fraction obtained when bone marrow cells are separated into populations of different cell types. The displacers, unlike salt, do not interfere with electrophoresis and protein fractions obtained by displacement can be directly subjected to electrophoresis or rechromatography without dialysis or concentration. Thus the examination of complex mixtures by a combination of chromatography and electrophoresis on a semi-micro scale is greatly expedited. The simplicity of operation also facilitates automation, so that the two-dimensional analysis of large numbers of fractions becomes feasible.

Moreover, since migration is induced by displacers under conditions of salt and pH in which the migrating molecules are otherwise strongly adsorbed, the possibility arises of chromatographing substances that are

"irreversibly" adsorbed in conventional procedures. National Cancer Program: objective 2, approach 2.

Proposed Course of Research: The possibility of imposing a pH gradient on the displacement process will be explored in order to reduce the charge density required for displacers capable of displacing proteins of very high affinity and to improve resolution. The search for suitable cationic displacers will continue. The method, as it develops, will be applied to the separation of proteins in several readily available tissue extracts, to the subfractionation of t-RNA, and to the examination of proteins and nucleic acids in guinea pig bone marrow cells.

Honors and Awards: None

Publications:

1. Yaron, A., Otey, M. C., Sober, H. A., Katchalski, E., Erlich-Rogozinski, S., and Berger, A.: Lysine oligopeptides. Preparation by ion exchange chromatography. Biopolymers 11: 607-621, 1972.

Serial No. NCI-333
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biochemical and immunochemical studies on normal and leukemic leukocytes

Previous Serial No.: Same

Principal Investigators: Warren H. Evans, S. Ralph Himmelhoch

Other Investigators: Michael G. Mage, Charles Grieshaber, Thomas Gessner, Freesia Huang, Emma Shelton, Margaret Wolf, and Shirley Wilson

Cooperating Units: Metabolism Section, Molecular Diseases Branch, NHLI

Man Years:
Total: 6
Professional: 6
Other: 0

Project Description:

Objectives: To investigate the biochemical factors regulating growth, maturation, and the phagocytic capacity of leukocytes, especially with regard to the arrested maturation and decreased phagocytic activity of leukocytes from myelogenous leukemia; to exploit any biochemical differences observed between normal and leukemic leukocytes for purposes of improving diagnosis and treatment of leukemia. The development of myeloperoxidase and alkaline phosphatase in the course of leukocyte maturation is being studied because of their favorable biological and physical properties. The chemical "structure" of the different classes of leukocyte granules is under investigation by chemical and immunological procedures.

Methods Employed: Leukocytes are isolated from bone marrow, blood, and inflammatory exudates by a variety of methods. Subcellular fractions are prepared by differential centrifugation and further fractionated by chromatography and electrophoresis. Immunological reagents, including fluorescent antibodies, are prepared from the purified fractions and used to follow the development of related material during maturation of the cells. The

maturation of enriched cell populations is studied in soft agar and stationary tissue culture.

Major Findings: Quantitative cytologic studies of the proliferation and maturation of guinea pig marrow neutrophil granulocytes have been carried out in short-term suspension cultures with a chemically-defined (serum-free) medium. An inverse correlation was observed between the absolute number of mature neutrophils in the initial marrow cell suspension and the increase in the number of these cells after 24 hours. In cultures of marrow cells from animals whose marrow had been depleted of mature neutrophils by an inflammatory stimulus in vivo, the absolute number of mature neutrophils increased 6-fold after 24 hours. No significant decrease in immature neutrophils was noted during the culture period, indicating that the observed maturation proceeded with cell proliferation. Concomitant with these changes in granulocytic cells, the absolute number of lymphocytes dropped to one-sixth their initial number, while the absolute number of other nucleated cells remained essentially unchanged. On addition of isologous dialyzed serum to the cultures of mature neutrophil-depleted marrow, the increase above starting levels in the absolute number of mature neutrophils was only one-half that observed in the absence of serum.

A search for granulopoeitic factors which might be released in response to the depletion of marrow granulocytes after leukapheresis has been undertaken. Analogous to studies which demonstrate that erythropoietin can be detected by its effect on hemoglobin synthesis in vitro, our studies suggest that granulopoietic factors in serum might also be detected by assays which measure the synthesis of specific granulocyte proteins in vitro. As a first step in the development of a biochemical assay for granulopoietic factors we investigated the effect of serum from normal and leukapheresed guinea pigs on amino acid -^{14}C incorporation into proteins from subcellular fractions of granulocyte-enriched bone marrow in vitro. In confirmation of preliminary results reported last year, we have now shown that, relative to fetal calf serum controls, normal serum enhances the incorporation of label into proteins associated with a slow sedimenting granule-containing fraction. Evidence has been obtained which suggests that the subcellular fraction affected by normal guinea pig serum is derived mainly from granulocytes. Amino acid incorporation into this fraction was significantly greater in cells incubated in the presence of serum from leukapheresed guinea pigs than in the presence of serum from normal guinea pigs. The factor(s) in serum responsible for these effects in non-dialyzable, and ion-exchange chromatography has shown that it is associated with only a small fraction of the serum protein.

Studies are being carried out on the incorporation of labeled glucosamine into cellular macromolecules of guinea pig bone marrow granulocytes. As

an extension of previous autoradiographic studies, investigations on the incorporation of tritiated glucosamine into the TCA-insoluble fraction of granulocytes separated from other bone marrow cells by velocity sedimentation at unit gravity confirmed that mature granulocytes (polymorphonuclears + bands) incorporate glucosamine at a much greater rate than immature cells. The major labeled compound in the hydrolysate of the TCA-insoluble fraction corresponded to hexosamine standard on paper chromatograms. Optimal incorporation of glucosamine appeared to be dependent upon the presence of previously synthesized protein and probably involved UDP-N-acetylglucosamine and N-acetylglucosamine as intermediates in the process of incorporation. The data suggest that glucosamine is incorporated into glycoprotein of bone marrow granulocytes during the later stages of cell maturation.

An investigation of human leukocytes (in collaboration with Drs. V. Manganiello, T. Stossel, R. Mason and M. Vaughn, NHLI) exposed to polystyrene latex beads showed that the cyclic AMP levels of such cells was increased several fold. Cell separation studies showed that preparations enriched in mononuclear cells (lymphocytes + monocytes) and containing only 0-20% granulocytes exhibited a quantitatively similar response. The response of fractions containing 80-90% granulocytes was only about one-sixth that observed with mononuclear cells. There was no change in the cyclic AMP content of guinea pig exudate granulocytes or of rabbit alveolar macrophages during phagocytosis of polystyrene beads. These observations are consisent with the view that particle uptake per se does not increase cyclic AMP levels in phagocytic cells.

A radioimmuno assay for neutrophil peroxidase is being developed in order to detect and measure neutrophil peroxidase with increased sensitivity. The solid phase consists of antibody to neutrophil peroxidase trapped in a macroporous polyacrylamide gel. The binding of the reference antigen (^{125}I-neutrophil peroxidase) is inhibited by varying amounts of unlabeled neutrophil peroxidase.

A protocol involving sensitization of strain 13 guinea pigs to Trichinella antigens has been devised that provides biochemically useful quantities of eosinophils for study. Improved methods for breaking eosinophils from these exudates which leave the granule fractions well preserved have been developed. A fraction of morphologically intact eosinophil granules uncontaminated by other subcellular fractions has been prepared and its properties confirmed by electron microscopic examination. The proteins of the granule have been quantitatively extracted and examined by chromatographic and gel electrophoresis methods for the first time.

Only three major protein components are detected in contrast to the more than thirteen "activities" associated with the granules by histochemical tests. One of these proteins has been identified as peroxidase. The "crystalloid inclusion" of the eosinophil granules has been liberated in intact form

and tentatively identified as the major protein component of the granule.

Human myeloperoxidase isolated as previously described has been characterized in detail with respect to physical homogeneity, molecular weight, and subunit structure. The molecule has been examined with the electron microscope. Human myeloperoxidase has been shown to have a native molecular weight of 139,000 and to consist of two subunits of identical size which appear as approximately spherical bodies under the E. M. No evidence for isozymes (reported by other workers) has been found.

We have shown that myeloperoxidase is a TPNH-oxidizing enzyme, and preliminary evidence indicates that it is the only such enzyme present in the guinea pig leukocyte. Since TPNH generation is a central requirement for phagocytosis, these data suggest that myeloperoxidase is involved in the biochemical basis of a central function of the leukocyte and call for a reinterpretation of available data on TPNH oxidation in white cells.

Significance to Cancer Research: Understanding of normal and leukemic maturation depends upon clarification of the molecular processes whereby the cell controls the appearance and disappearance of specific components. Such information has never been obtained in a systematic fashion similar to that achieved in morphological studies of normal and leukemic granulocytes. National Cancer Program: objective 2, approach 3.

Proposed Course of Research: We wish to establish as detailed an understanding of the nature and order of the biochemical events of maturation as has previously been acheived at the morphological level. A detailed description of the changes in the biochemical constituents of nuclei, cytoplasm, cell membrane, and granules at various important stages of white cell development will be attempted in order to identify possible sites of hormonal and genetic regulation in both normal and leukemic cells.

Honors and Awards: None

Publications:

1. Manganiello, V., Evans, W. H., Stossel, T. P., Mason, R. Jr., and Vaughan, M.: The effect of polystyrene beads on cyclic 3, 5'-adenosine monophosphate concentration in leukocytes. J. Clin. Invest. 50: 2741-2744, 1971.

2. Desser, R. K., Himmelhoch, S. R., Evans, W. H., Januska, M., Mage, M., and Shelton, E.: Guinea pig heterophil and eosinophil peroxidase. Arch. Biochem. Biophys. 148: 452-465, 1972.

3. Grieshaber, C. K., and Evans, W. H.: Effect of serum on amino acid incorporation into protein of guinea pig bone marrow granulocytes. J. Reticuloendothel. Soc. In press.

Serial No. NCI-5203
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Development of immunochemical and physical methods for the purification and characterization of immunocytes and their subcellular components.

Previous Serial No.: Same

Principal Investigator: Michael G. Mage

Other Investigators: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: The goal of this project is the development of methods for the isolation of immune cells from the non-immune cells of lymphoid tissue, for the subfractionation of the various classes of immunocytes, and for their subcellular fractionation in order to study the mechanisms involved in the development of the different immunological activities of antibody proteins.

Methods Employed: To isolate polyribosomes, rabbits are immunized in the footpads to stimulate immunoglobulin production, and the popliteal lymph nodes are removed after labelling with radioactive amino acids and/or nucleosides. The tissue is disrupted and the polysomes are isolated by sedimentation in sucrose gradients. Anti-Ig antibodies used to precipitate polysomes are purified on immunoadsorbents.

Studies on cells giving a graft-versus-host (GVH) reaction have utilized the polyurethane foam immunoadsorbents previously developed in this laboratory. We have attempted to bind GVH-reactive lymphocytes from one inbred strain of mice to a foam disk coated with erythrocytes or lymphocytes from a second strain of mice. GVH activity is measured by the Simonsen spleen-weight assay in neonatal F_1 hybrids of the two strains used. (The two strains (BALB/c and C57/B16) have a strong H-2 histo-incompatibility).

Major Findings: We have developed an assay that measures by direct binding of ^{125}I-antigen, the specificity of the anti-immunoglobulin reagents used in the ribosome precipitation experiments. The assay, which utilizes anti-immunoglobulin antibodies trapped in a polyacrylamide gel, is being used to explore the non-specific precipitation of polyribosomes in the presence of anti-immunoglobulin.

In the GVH cell separation experiments, cells with GVH activity were found to survive the separation procedure at 37° and at 4°. However, the cells that bind to the RBC-foam are depleted of GVH activity. This result (which is diametrically opposed to our working hypothesis that GVH-reactive cells would be enriched in the bound fraction) may nonetheless indicate a potential way to separate GVH-reactive and non-reactive cells. The depletion might be due to inactivation of the GVH-reactive cells subsequent to binding, or to preferential binding of GVH-non-reactive lymphocytes.

Significance to Cancer Research: The studies on the precipitation of polyribosomes are intended to develop methods for the isolation of immunoglobulin -m RNA for molecular studies of immunocyte differentiation and of the basis of antibody variability. These studies, together with the GVH studies, which relate to the mechanisms of cell-mediated tumor rejection, may eventually aid in understanding the variation in tumor susceptibility of human beings and make possible control of immune mechanisms of tumor surveillance and rejection. National Cancer Program: objective 2, approach 1.

Proposed Course: We plan to improve our methodology for the separation of immunoglobulin-producing polysomes, to work on the isolation of their m-RNA, and on using this m-RNA in hybridization experiments. The cell separation experiments will be continued in order to develop a specific separation of GVH-reactive cells.

Honors and Awards: None

Publications: None

Serial No. NCI-5202
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Isolation, fractionation, and characterization of native nucleoproteins

Previous Serial No.: Same

Principal Investigator: O. Wesley McBride

Other Investigators: Harvey L. Ozer, Elbert A. Peterson

Cooperating Units: None

Man Years:
Total : 2
Professional: 1
Other: 1

Project Description:

Objectives: Determination of the role of nucleic-acid bound proteins in the regulation of the transcription and translation of genetic information and biological transformation of cells with isolated and fractionated native mammalian genetic material as a means of genetic mapping.

Methods Employed: Tissue culture methods are employed for the propagation of mammalian cells and induction of parasynchronous cultures, and cells are radioisotopically labelled with nuclei acid and protein precursors. Metaphase chromosomes are isolated from cell cultures arrested at mitosis, partially purified by differential and isopycnic centrifugation, and separated from nuclei and further subfractionated, according to mass, by unit gravity zonal sedimentation. The MW of chromosomal DNA is determined by velocity sedimentation in alkaline sucrose density gradients and liquid scintillation counting of the collected fractions. The uptake of ^{3}H-thymidine labelled chromosomes is assessed by scintillation counting of lysates of washed recipient cells. Biological transformation is detected by colonial growth of transformed mutant (HGPRTase^{-} or TK^{-})cells in selective media after ingestion of metaphase chromosomes isolated from wild type cells. The transformed colonies are cloned and further characterized by

growth in selective media, karyography, reversion rates in non-selective media, and identification of the gene products by cellulose ion-exchange chromatography, electrophoresis, and immunological methods. Phase and bright field microscopy and photomicroscopy as well as autoradiographic procedures are utilized.

Major Findings: The optimal conditions for ingestion of isolated metaphase chromosomes by cells have been established previously. A large fraction of the ingested chromosomal DNA is degraded to low MW ($< 10^6$) material within a few hours. Cells which are biologically transformed by ingested chromosomes have been detected at a low, and somewhat variable, frequency of about 10^{-6} by growth in selective media and this appears to be greater than the reversion rate of recipient cells in control cultures. The possibility of reversion was explored and excluded by physical characterization of the gene product (HG PRTase) from three unequivocally separate colonies isolated in one experiment employing Chinese hamster (CH, wild type) donor chromosomes and mutant (HG PRTase$^-$) recipient mouse L cells. The HG PRTase from each of these transformant clones is completely indistinguishable chromatographically (DEAE-cellulose) and electrophoretically from the same enzyme isolated from CH cells whereas it clearly differs from mouse L cell HGPRTase (wild type or TK$^-$ mutant) by both of these criteria. A rare revertant was finally isolated from control experiments, and the enzyme from this revertant line is identical to wild type mouse enzyme by the criteria above, as expected.

Although these three biologically transformed clones all produce the same HG PRTase gene product specified by the ingested chromosomes, they all differ from each other by additional criteria. Karyography exhibits 2-3 extra metacentric chromosomes in each of these clones but loss of these chromosomes accompanies loss of enzyme activity only in clone 1 and it also has a very stable phenotype in non-selective media ($<< 1\%$ HG PRTase$^-$ after several months). Clone 3 is quite unstable ($\sim 3\%$ HGPRTase$^+$ cells after 1 month) and clone 2 has intermediate stability. It is tentatively postulated that these results reflect differences between the rate of loss of an intact chromosome(s) and that of a chromosome fragment bearing the HG PRTase locus.

Biological transformants have been detected also employing different recipient cells (CH HG PRTase$^-$), different donor chromosomes (human HeLa), and a different selective marker system (thymidine kinase). None of these other lines have been characterized further to exclude the possibility of revertants, however.

Proposed Course of Research: The general applicability of biological transformation by isolated metaphase chromosomes to the problem of genetic mapping will be demonstrated by obtaining further evidence that other (non

X-linked) genes, such as the gene for thymidine kinase, are also expressed. This requires physical characterization of the product by means comparable to those used for HG PRTase.

Studies are in progress to determine whether the entire X-chromosome is present in these clones and whether it is intact. We are examining these clones for the expression of other X-linked genes (G-6PD and phosphoglycerate kinase) of the CH donor type and, if present, whether they are lost concomitantly with HG PRTase (persistent linkage) or independently, indicating chromosome fragmentation or translocation. We are also collaborating with Dr. M. Mage in looking for CH surface antigens on these cells since this is a function coded for by several genes on more than one chromosome. Collaboration with Dr. Thirion is underway to identify a CH chromosome in these cells by the fluorescent banding technique of Casperson. Dr. H. Green has provided an HG PRTase$^-$ line of mouse 3T6 cells lacking metacentric chromosomes, so subsequent experiments involving these cells should permit very simple identification of an incorporated CH chromosome.

A fundamental problem in making this a useful method for human genetic mapping is to discover methods to increase the frequency of biological transformation. Probably the rate-limiting step is transfer of the chromosome from the cytoplasm to the nucleus in the absence of extensive lysosomal degradation of the chromosomal DNA. Several reasonable approaches are under consideration to reduce or circumvent this problem. Methods to fractionate chromosomes further will also be explored.

Significance to Cancer Research: Metaphase chromosome isolation and fractionation provides the most promising approach for the fractionation of mammalian genes and can provide material for the investigation of control elements associated with specific sets of genes. The biological expression of these chromosomes introduced into recipient cells provides a method for mapping of mammalian genes, investigating differences between genetic elements in normal and neoplastic cells, and looking for sites of integration of oncogenic viruses into the mammalian genome. National Cancer Program: objective 2, approach 3.

Honors and Awards: None

Publications:

Serial No. NCI-380
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Deoxyribose containing compounds in normal, regenerating and cancer tissues.

Previous Serial No.: Same

Principal Investigator: Walter C. Schneider

Other Investigators: William Kidwell, Gilbert Smith

Cooperating Units: Laboratory of Biology

Man Years:
Total: 3
Professional: 1
Other: 2

Project Description:

Objectives: Since the replication of DNA is an obligatory prerequisite for cell division, the study of DNA synthesis and its control occupies a position of unique importance in cancer research because the malignant cancer cell often displays continuous and uncontrolled division. The objectives of this project are to isolate and identify deoxyribose-containing compounds other than DNA from normal precancerous and tumor tissues, to determine the metabolic sequences in which these compounds are involved and whether these sequences are the same in normal precancerous and cancer tissues, to isolate DNA from subcellular structures other than nuclei, to study the physical and biochemical properties of extranuclear DNA and to determine the relationship between nuclear and extranuclear DNA in carcinogenesis.

These studies are aimed at uncovering differences between normal and malignant cells that can be exploited to prevent the formation of cancer cells or to promote the destruction of cancer cells already generated. (These studies support most closely Objective 3: To prevent conversion of cells to those capable of forming cancer and Approach 1: Nature and modification of the precancerous state, of the National Cancer Plan).

Methods: Nuclei, mitochondria, zymogen granules, and microsomes were isolated from rat or mouse tissues by differential and/or density gradient

centrifugation. Nucleic acids were extracted from tissue fractions using sodium lauryl sulfate and phenol. DNA was purified from these extracts by treatment with ribonuclease and passage through Biogel P-60 or by banding in CsCl.

DNA samples were examined by means of analytical CsCl density gradient centrifugation, melting behavior, and amount of labeled thymidine incorporated. DNA was measured by a modification of the diphenylamine reaction.

Enzyme markers were used for estimating purity of isolated subcellular fractions. Reduction of INT (iodonitrotetrazolium violet) in the presence of succinate or DPNH was used as a measure of mitochondria or microsomes, respectively. Amylase was used as a measure of zymogen granules in the pancreas.

Major Findings: During the past year a number of tissues other than liver were fractionated to determine if they also contained microsomal associated DNA. Spleen, kidney, brain, pancreas, submaxillary glands, and a plasma cell tumor were examined and all were found to contain DNA in the microsomal fraction. The amounts of microsomal DNA present were small in all cases but of the same magnitude as found in liver. The specific activity of the microsomal DNA was usually greater than that of nuclear DNA except in the case of brain and the plasma cell tumor where little difference was observed.

The presence of DNA was also sought in another subcellular organelle, the zymogen granules of the pancreas. This proved to be a more difficult task than anticipated because the zymogen granules, nuclei and mitochondria did not separate cleanly. Encouraging results were obtained with sucrose density gradients in centrifuge tubes but a really excellent separation was achieved in the zonal centrifuge rotor, which has finally become operational after about a 2 year delay. With this rotor zymogen granules, essentially free of mitochondria, were obtained in high yield. The amount of DNA present in the granules was very low but further experiments will be required to determine if they contain any DNA at all.

A considerable amount of time was spent this year automating various methods for use with the zonal ultracentrifuge. Methods using Technicon equipment have been developed for protein, succinic dehydrogenase, DPNH-cytochrome C with reductase, glucose-6-phosphatase, and measurement of optical density. These methods have already proved their value in preliminary work with the zonal ultracentrifuge.

A simpler method of DNA isolation developed at the Carnegie Institute was adapted to mitochondrial and microsomal fractions. In this method the fractions are dissolved in a mixture of urea, sodium lauryl sulfate, EDTA, and phosphate buffer and passed through a hydroxyl-apatite column. Proteins and RNA are excluded while DNA is retained and subsequently sharply eluted at a higher concentration of phosphate.

Results on DNA-DNA hybridization indicate that rat liver microsomal DNA hybridizes to the same extent with nuclear DNA as nuclear DNA hybridizes with itself. Hybridizations involving liver mitochondrial DNA have not been carried out as yet.

Additional experiments on ovaries (with Drs. Kidwell and Smith) aimed at determining whether microsomes were produced during superovulation were carried out but have not led to any clearcut indication of increased microsomal DNA activity.

Significance of Findings: The results of the tissue fractionation studies show that DNA was associated with the microsomal fraction of all the tissues examined including one tumor. This DNA could be differentiated from nuclear DNA on the basis of thymidine incorporation in all the tissues examined except for brain and the plasma cell tumor. The latter two tissues represent extremes in DNA synthesis in that brain has little or none while the tumor has a very high rate and this may accouht for the failure of the incorporation into microsomal DNA to differ from that into nuclear DNA. At any rate, the results show that microsomal-associated DNA is a widespread phenomenon. The real significance of this observation must await the establishment of a function for this DNA and the question of how this DNA might be involved in carcinogenesis also remains undecided.

The DNA-DNA hybridization results indicate that the microsomal DNA was closely related to the nuclear DNA and may mean that the microsomal DNA originated in the nucleus and serves a messenger role. Such a transport of DNA from nucleus to cytoplasm remains to be demonstrated, however.

Preliminary experiments with the zonal ultracentrifuge rotor indicate that an exceedingly sharp separation of zymogen granules was achieved and that the separation surpassed what could be done by differential centrifugation. This tool should permit fractionation of tissue suspensions into sharp fractions in a single step and should avoid artifacts caused by washing, sedimentation under non-ideal conditions, and aggregation.

Proposed Course of Research: The success achieved with the zonal rotor in the isolation of zymogen granules leads one to hope that similar success will be enjoyed for the isolation of other subcellular organelles. It is planned to use this method almost exclusively during the coming year for the isolation of tissue fractions and to determine its possibilities. Again the emphasis will be upon whether DNA can be found in subcellular organelles such as the zymogen granules, melanin granules, etc., and if so to characterize the DNA and determine its properties.

The DNA-DNA hybridization experiments will be continued and hopefully concluded during the coming year.

Serial No. NCI-5211
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Initiation and Maturation phenomena in the replication of mammalian DNA

Previous Serial No.: Same

Principal Investigator: William R. Kidwell

Other Investigators: R. E. Scott, NIAMD-LEP; R. Saral and R. E. Martin, NIAMD-LMB; H. L. Ozer, NCI-LB.

Cooperating Units: Laboratory of Experimental Pathology and Laboratory of Molecular Biology, NIAMD

Man Years:
Total: 3
Professional: 1-1/2
Other: 1 1/2

Project Description:

Objectives: The cells in most tissues and organs of higher species accumulate in a non DNA replicative stage of the cell cycle denoted as G_0 or extended G_1. In malignancy there is characteristically an increase in the rate of cell proliferation and the normal regulatory mechanism leading to an accumulation of cells in G_0 or extended G_1 is lost. This investigation places emphasis on elucidating the mechanism of initiation of DNA synthesis, a rate limiting process in normal cells which occurs with accelerated frequency in malignancy.

Three aspects of the project were: 1) To characterize the initiation of replication of a particular type of DNA, satellite DNA of L-cells which can be separated by physical means from the bulk DNA of the nucleus; 2) Explore the mechanisms whereby cells accumulated in extended G_1 (contact inhibited) are induced to undergo DNA replication by viruses; 3) Examine changes that occur in the membranes of cells during the progression through the various stages of the cell cycle, particularly through G_1 into S.

Methods Employed: To study the initiation phenomenon, an *in vitro* DNA synthesizing system utilizing mouse L-cells nuclei was developed. The differential affect of cytoplasmic extracts of cells at various stages of S on

satellite and bulk DNA synthesis was assessed by fractionation of the two DNA species on hydroxyapatite following denaturation and reassociation.

SV40 virus propogated on BSC, CV-1 or Vero cells was purified by polyethylene glycol extraction followed by banding to equilibrium several times in CsCl.

Changes in the membranes of cells at various stages of the cell cycle were examined electron microscopically following replica plating of freeze-cleaved cells. Synchrony was attained by shake selection or by use of inhibitors of DNA replication

Results: Initiation of satellite DNA synthesis: Utilizing the in vitro DNA synthesizing system described earlier, cytoplasmic factors have been detected in cells which differentially affect the labelling of satellite DNA. These factors appear to be present only during the time when cells are actively engaged in the synthesis of satellite DNA. Cytoplasmic extracts from such cells have been fractionated on hydroxyapatite and two species which stimulate satellite DNA synthesis have been detected.

Detection of an endonuclease activity in purified SV40 virions: Virus induced transformation of cells in tissue culture and consequently the initiation of host cell DNA replication is well documented. However, purified viral DNA is less capable of inducing the transformed state (Cuzin) suggesting that an additional viral component is required. A postulated candidate in endonuclease has been detected in SV40 virions. The activity is heat labile and has an absolute requirement for a divalent cation. It has been demonstrated to be intimately associated with the viral particles by: 1) Cobanding with SV40 virions (full and empty particles) in CsCl; and 2) By neutralization of endonuclease activity with specific viral antisera. Virus purified by three different techniques possesses endonuclease activity. Three different temperature-sensitive SV40 mutants (Ts 101, TsA7, and TsA8) have endonuclease activity when assayed at the permissive and nonpermissive temperature. Disruption of the virions (high pH or with detergents) led to nearly complete loss of endonuclease activity. Kinetic studies of the product of the endonuclease activity indicate that the enzyme initially produces a single strand break in closed circular DNA and subsequently leads to a limit product DNA with an S value of 10-13. Single strand breakage was verified with the electron microscope. Closed twisted circular DNA substrate was seen to be converted to relaxed circles. This work was performed in collaboration with H. L. Ozer (now of the Worcester Foundation for Experimental Biology, Schrewsbury, Mass) and R. Saral and R. Martin (LMB-NIAMD).

Changes in cell membranes during the cell cycles: Freeze-cleavage of biological membranes fractures cell membranes in half exposing two inner fracture faces. These fracture faces are partially covered with 70 Å particles which are apparently involved in the binding of certain viruses and

possibly, plant lectins. In a study with R. Scott (LEP-NIAMD) the density of the 70 Å particles (number of particles per unit area) on the fracture faces of Chinese hamster and L-929 cells was found to change during the cell cycle. A high density of particles was observed on the fracture faces of cells in late G_1 through metaphase. An abrupt 2-3 fold decrease in particle density occurred during the transition from metaphase to early G_1. Particle density increased rapidly during G_1 and tended to plateau with the approach of the next S-phase. Concomitant increases in the density of pores on nuclear membranes were seen during the G_1 interval. Examination of the fracture faces from contact inhibited cells and cells released from contact inhibition by trypsinization revealed no change in the number of particles per unit area but rather a unique clustering of the particles in contact inhibited cells instead of the random distribution seen in all other cases.

Significance to Cancer Research: Central to the question of increase of cell proliferation in the malignant state is an understanding of the factors governing control mechanisms which normally lead to the accumulation of cells in a G_0 or extended G_1 stage of the cell cycle. What is certainly involved ultimately is the initiation of DNA replication. Two probes which may lead to an understanding of this pehnomenon are studies on factors controlling the initiation of satellite DNA synthesis and the endonuclease associated with SV40 virions (which may be involved in triggering DNA synthesis in the appropriate host cell). Probably the least characterized are the changes in cells during G_1 which precede DNA replication. In this study we have demonstrated dramatic changes in the density of particles on the fracture faces of membranes during G_1. While the significance of these changes is unknown, it is generally true that membrane function and particle density are directly related. National Cancer Program: objective 3, approach 1 and 5.

Proposed Course of Research: Continued effort will be directed toward characterization of the factors in L-cell cytoplasm which affect satellite DNA replication. In addition, we will attempt to demonstrate the role of the viral associated endonuclease by screening viral mutants in hopes of detecting such a mutant whose endonuclease activity is temperature-sensitive. If such a mutant is detected it will be checked to see if host cell DNA synthesis is turned off at the nonpermissive temperature.

Honors and Awards: None

Publications:

1. Scott, R. E., Carter, R. L., Kidwell, W. R.: Structural changes in membranes of synchronized cells demonstrated by freeze-cleavage. Nature-New Biology 233: 219-220, 1971.

2. Kidwell, W. R.: Fidelity of DNA replication in isolated L-cell nuclei. Biochim. Biophys. Acta. In press.

Serial No. NCI-5205
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on biochemical mechanisms of sensitivity and resistance to antimetabolites and mechanisms for control of nucleic acid synthesis

Previous Serial No.: Same

Principal Investigator: Elizabeth P. Anderson

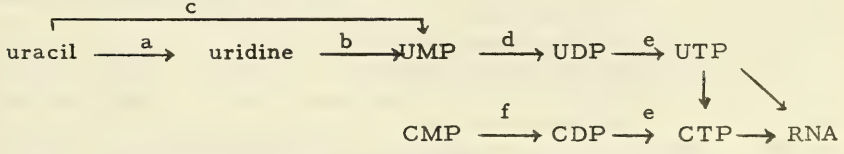
Other Investigators: Thomas Q. Garvey, III, and Alec S. Liacouras

Cooperating Units: F. K. Millar, Laboratory of Physiology, NCI

Man Years:
Total: 4
Professional: 3
Other: 1

Project Description:

Objectives: The objective of this project is to investigate nucleotide metabolism in normal and cancer cells. The aim is to elucidate pathways of biosynthesis, mechanisms of control over these pathways, and biochemical mechanisms of sensitivity and resistance to purine and pyrimidine analogs. The end-products of nucleotide biosynthesis are the triphosphate precursors of nucleic acids, and regulation of the enzymes involved in these biosynthetic steps is probably a major factor in determining the rate of cellular nucleic acid synthesis. In view of the central role of UMP in pyrimidine metabolism work has been focussed on reactions b, d, and f in the following pathway of pyrimidine nucleotide biosynthesis:



This project most closely supports objective 3, approach 2, of the National

Cancer Program.

Methods Employed and Major Findings:

1) Studies on UMP-CMP kinase. Further studies have been carried out on UMP-CMP kinase, the enzyme that catalyzes reactions d and f above. These studies have been performed with 300-fold purified preparations of the enzyme from Tetrahymena pyriformis; this is a rapidly growing protozoan unable to synthesize the pyrimidine ring de novo and hence dependent on pyrimidine anabolism from the preformed ring. The reactions catalyzed by the enzyme are:

$$\begin{matrix}\text{UMP} \\ \text{or} \\ \text{CMP}\end{matrix} + \text{ATP} \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} \begin{matrix}\text{UDP} \\ \text{or} \\ \text{CDP}\end{matrix} + \text{ADP}$$

Using a refined radioactivity assay to measure the phosphorylation of C^{14}-UMP, initial velocity studies have been carried out to explore the details and mechanism of this kinase reaction with UMP as substrate. These studies have produced data consistent with a sequential mechanism, involving the formation of a ternary complex of the enzyme with both substrates as the central intermediate in the reaction. The initial velocity pattern also indicates that the reaction is subject to competitive bisubstrate inhibition, suggesting similarity of the binding sites for the two substrates. In addition, studies have been made on isotope exchange at equilibrium. The purified enzyme has been found to be slightly contaminated with nucleoside diphosphate kinase, which results in transphosphorylation between ATP and ADP over long time periods. Nevertheless, the isotope exchange data rule out a classical "ping pong" reaction mechanism in which one of the reactants adds to, and one product is released from, the enzyme prior to interaction of the modified enzyme with a second reactant.

Investigations of the kinase reaction have now also been undertaken with CMP as the phosphate acceptor. With this substrate a less complicated initial velocity pattern has been observed, and the evidence for a sequential reaction mechanism is even more clear-cut. These studies have been facilitated by the fact that the apparent K_m of the enzyme for CMP is about 0.3 mM, in contrast of the figure of 1.0 mM for UMP. Also, it has been possible to assay the reaction with CMP as substrate using a continuous spectrophotometric assay that couples the production of ADP to the pyruvate kinase - lactate dehydrogenase system.

These studies have been carried out by Dr. Thomas Garvey. In addition to the biochemical studies, he has made use of the IBM 1620, the IBM 360, and the PDP-10 computers for routine calculations and for the more complicated problems of curve fitting and statistical evaluation of the data. In these computer operations, he has collaborated with Dr. F. K. Millar,

Laboratory of Physiology, NCI. The results of the studies with UMP kinase are now being prepared for publication.

2) Studies on uridine kinase. In mammalian tissues uridine kinase (reaction b above) is the initial anabolic step in pyrimidine nucleotide biosynthesis from the preformed ring. This reaction has been shown to be rate-limiting in the biosynthetic pathway and susceptible to feedback inhibition by nucleoside triphosphate end-products. This enzyme has now been purified approximately 200-fold from acetone powder preparations of the mouse ascites mast cell neoplasm P815/S; this has been achieved using fractionation on DEAE-cellulose at pH 7.5, ammonium sulfate fractionation, separation on hydroxylapatite, and fractionation on DEAE-cellulose at pH 8.2. The stability of the enzyme after the second DEAE-cellulose step is only fair, and the effectiveness of various stabilizing agents is currently under investigation. After the hydroxylapatite step, the enzyme was found to exhibit at least six bands in polyacrylamide gel electrophoresis. Two assay systems are presently being used for this enzyme reaction; one measures the conversion of C^{14}-labeled uridine to nucleotide product, while the second, which can only be used on more purified fractions, assays the production of ADP by coupling this to the pyruvate kinase-lactate dehydrogenase system and spectrophotometrically determining the conversion of NADH to NAD. Analyses have shown that the sensitivity to feedback inhibition by CTP has been retained by the enzyme through these purification steps. These studies have been carried out by Mr. Alec Liacouras.

3) Related activities. Work is in progress on an extensive review of nucleoside and nucleotide kinases for the new edition of The Enzymes. This will cover not only the two enzymes on which research research is currently in progress, but also related nucleoside kinases and nucleoside monophosphate kinases. This will go to press in the summer of 1972.

Significance to Cancer Research: UMP is the central compound in pyrimidine nucleotide biosynthesis in that it is the product of pyrimidine synthesis de novo and thereby the precursor for synthesis of other pyrimidine nucleotides of both the ribo- and deoxy-ribo series. Undoubtedly much UMP is synthesized through the pathway of ring synthesis de novo, but nucleotide biosynthesis from preformed rings is also called into play in rapidly growing tissue and the enzymes of these pathways exhibit increased activity in such tissues. It is important to discover how the various enzymes for UMP synthesis and metabolism are regulated in normal and cancer cells, since such regulation undoubtedly helps to maintain a balance among the levels of the various nucleotide precursors of nucleic acids and functions in regulating the rate of cellular nucleic acid synthesis.

Proposed Course of Research: In the studies on UMP-CMP kinase, additional experiments will be performed to measure the initial velocity of isotope

exchange at equilibrium with the UMP-UDP and ATP-ADP substrates, as well as the long-term exchange, and similar studies will also be carried out on the isotope exchange with the CMP-CDP and ATP-ADP substrates. Product inhibition studies are also planned to establish whether the addition of substrates to the enzyme to form the ternary complex is random or occurs in an ordered fashion, and if ordered, what the order of addition is.

The purification of uridine kinase from the mast cell neoplasm will be continued with the aim of stabilizing the enzyme at its present high level of purification. This should make it feasible to explore the molecular nature of the enzyme protein, particularly with regard to its molecular weight and possible subunit structure. The mechanism of its feedback inhibition will then be investigated in terms of the nature of the enzyme molecule and its catalytic and regulatory sites.

Honors and Awards: None

Publications: None

Serial No. NCI-397
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies comparing the structure, properties and metabolism of the nucleic acids and nucleoproteins of cultured cells and of tumors derived from them.

Previous Serial No.: Same

Principal Investigator: Jean Rotherham

Other Investigators: None

Cooperating Units: Tissue Culture Section, Laboratory of Biology, NCI

Man Years: (not applicable)

Project Description:

Objectives: To discover if there are changes in structure, properties or metabolism of the nucleic acids or nucleoproteins of cultured mammalian cells which are characteristic of and associated with transformation from pre- to post-malignant stages.

Methods and Major Findings: Some aspects of the role of vitamin B_{12} and folic acid in DNA-thymidine synthesis in a neoplastic murine cell strain, NCTC 5905.

We found that a function of vitamin B_{12} seems to be, in 5905 cells in culture, that of increasing DNA-thymidine synthesis by raising limiting levels of folate coenzymes that are required in the metabolism of "one-carbon fragments". Also some other suggested functions seem to be inoperative as judged by these results and those we have published previously.

Some additional data related to our previous studies concerning other aspects of the metabolism of nucleic acid precursors in cells of this strain were obtained.

Significance: The results described have contributed to a more complete understanding of some aspects of metabolic reactions by which these murine neoplastic cells synthesize DNA-precursors that are needed for continuous proliferation in culture. National Cancer Program Plan Objective 3, Approach 2.

Proposed Course of Work: This project has been interrupted because the biological material was not available under workable conditions. It is propose that it be continued.

Honors and Awards: None

Publications:

1. Rotherham, J., Price, F. M., Otani, T. T., and Evans, V. J.: Some aspects of the role of Vitamin B_{12} and folic acid in DNA-thymidine synthesis in a neoplastic C3H mouse cell strain. J. Nat. Cancer Inst. 47: 277-287, 1971.

Serial No. NCI-5220
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies comparing the structure, properties and metabolism of the nucleic acids and nucleoproteins of mammary tumors in rats.

Previous Serial No.: None

Principal Investigator: Jean Rotherham

Other Investigators: None

Cooperating Units: Tumor Physiopathology Section, Laboratory of Biochemistry, NCI

Man Years:
Total: 1/3
Professional: 1/3

Project Description:

Objectives: To discover if there are differences between regressing and growing hormone-dependent tumors and between growing hormone-dependent and hormone-independent tumors with respect to the structure, properties, or metabolism of the nucleic acids and nucleoproteins of mammary tumors in rats.

Methods: Methods presumed to be suitable for isolation of nuclear fractions from the tissue and for study of their biosynthetic properties are being examined.

Findings: This study is in its initial plans

Significance: See Objectives. National Cancer Program Plan Objective and Approach: Cause and prevention; nature and rate of oncogenic cell transformations in carcinogenesis (3, 2).

Honors and Awards: None

Publications: None

Serial No. NCI-394
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on isozyme distribution in various transplantable rat hepatomas.

Previous Serial No.: Same

Principal Investigator: Theodore T. Otani

Other Investigators: Harold P. Morris

Cooperating Units: Cancer Research Unit, Department of Biochemistry, Howard University, College of Medicine, Washington, D. C.

Man Years:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: To expand basic understanding of the natuee of malignancy by studying the role of isozymes of several enzymes on the physiology of the cell and to correlate this role to the problem of neoplasia. More specifically, the aim of this portion of the project is to seek a relationship between activities and also isozyme distribution of four enzymes and the growth rates of certain tumors.

Significance to Cancer Research: This project supports Objective 4, Approach 2 of the National Cancer Plan.

Honors and Awards: None

Publications: Otani

1. Otani, T. T., and Morris, H. P.: Comparison of activity and isozyme patterns of four enzymes from hepatomas of different growth rates. J. Nat. Cancer Inst. 47: 1247-1253, 1971.

Serial No. NCI-306
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of β-hydroxy-α-amino acids

Previous Serial No.: Same

Principal Investigator: Theodore T. Otani

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: To prepare chemical compounds which preferentially destroy cancer cells. The preparation of β-hydroxy-α-amino acids and their N-acylated derivatives is being attempted, since earlier experiments done in this laboratory indicated that some compounds of this group are capable of inhibiting bacterial as well as tumor growth.

More specifically, the immediate objectives of this portion of the project are: (a) To isolate the four isomers of β-hydroxynorleucine prepared in this laboratory, and also to prepare their respective N-acyl derivatives in sufficient quantities for study of their possible tumor inhibitory action in mammalian systems.

(b) To prepare the analogs of methionine, i.e., β-hydroxyhomomethionine, β-hydroxymethionine, and "β-hydroxy-oxymethionine" (γ-methoxy-β-hydroxy-α-amino-n-butyric acid) from glycine copper complex and the appropriate aldehydes.

(c) To prepare various halo-substituted aliphatic β-hydroxy-α-amino acids from halo-alkyl aldehydes and glycine copper complex.

(d) To prepare β-hydroxy-α-asparagine, and β-hydroxy-β-asparagine from glycine amide and glyoxylic acid, and from glyoxylic acid amide and glycine respectively.

Methods: The preparation of these compounds are carried out in alkaline medium with the appropriate aldehyde and glycine copper complex (or glycine and cupric ions) under conditions which are empirically determined.

Major Findings:

1. The four isomers of β-hydroxynorleucine were isolated by separating the diasteromers using a cellulose column, and resolving the optical isomers by asymmetric enzymatic hydrolysis of the N-acyl derivatives.

2. N-Acyl-L-amino acids were prepared by acylation of the free L-amino acids. The N-acyl-D-amino acids were isolated from the digest of the asymmetric hydrolysis, as this isomer remained unhydrolyzed by the enzyme

3. β-Hydroxyhomomethionine has now been prepared in pure crystalline state. The procedure involved the initial removal of salt from the reaction mixture by passage of the solution through a cation exchange column, and the isolation of the products by the passage of the desalted solution through a cellulose column.

4. β-Hydroxy-oxymethionine has also been prepared in crystalline state. Thus far, the racemic diastereomer A and the racemic diastereomer B of both β-hydroxyhomomethionine and β-hydroxy-oxymethionine have been isolated.

Significance: That amino acid metabolism may be a susceptible area of action in tumor therapy was demonstrated by the efficacy of asparaginase in certain tumors which required asparagine. Amino acid deprivation, either by the removal of the amino acid or by substitution with an analog, may be effective in inhibiting tumor growth.

The un-natural amino acids synthesized under this program are close structural analogs of essential natural amino acids, differing in some cases only in the presence of a single hydroxyl group adjacent to the α-amino group. Hence, β-hydroxynorleucine differs from norleucine only in the presence of a hydroxyl group in the β-position of carbon chain of the amino acid. β-Hydroxynorleucine is a structural analog of methionine, differing from the latter in having a methylene group in place of the sulfur atom in the γ-position of the chain. Indeed, β-hydroxynorleucine, even as a mixture of four isomers, not only exhibited inhibitory action in two microbial systems, but also showed some anti-tumor effect against Carcinoma 755 as measured by the decrease of tumor size and increase in survival time. β-Hydroxy-oxymethionine and β-hydroxymethionine are even closer analogs of methionine than is β-hydroxy-homomethionine, the former differing from methionine in possessing an oxygen atom in place of the S atom and in processing a β-hydroxyl group,

and the latter only in possessing a β-hydroxyl group.

The chain-halogenated amino acids, similarly, are close structural analogs of essential amino acids. For example, γ-dichlorothreonine would be an analog of leucine, since the size of the chlorine atom is approximately that of the methyl group; also γ-triflurothreonine would be a close analog of threonine since the atomic radius of the fluorine atom is essentially that of hydrogen atoms. Objective 6: Approach 2 - National Cancer Program.

Proposed Course of Study:

1. The four isomers of β-hydroxynorleucine and their corresponding N-acyl derivatives will initially be tested against microbial anti-tumor screening systems to determine the presence of possible anti-tumor activities. The compounds showing growth-inhibitory action against the screen will then be further tested against representative tumors in mice.

2. Large amounts of each of the four isomers of β-hydroxynorleucine and the corresponding N-acyl derivatives of each isomer will be prepared and will be tested in mammalian tumor systems.

3. Chemical identification and proof of structure will be made on the new compounds. β-hydroxyhomomethionine and β-hydroxy-oxymethionine.

4. The four isomers of each of these methionine derivatives will be isolated and the N-acyl derivatives of each will be made. All eight of these compounds of each methionine derivative will be tested for anti-tumor activity against a microbial anti-tumor screening system, and the compounds found to show growth-inhibitory activity in the screen will ultimately be tested in representative systems of mammalian tumors.

5. Attempts to prepare β-hydroxymethionine, halo-substituted β-hydroxy-amino acids and the β-hydroxyasparagines will be continued.

Honors and Awards: None

Publications: None

Serial No. NCI-366
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biosynthesis and assembly of intracellular structural components.

Previous Serial No.: Same

Principal Investigator: Edward L. Kuff

Other Investigators: Kira K. Lueders, Harvey L. Ozer, Nelson A. Wivel, Samuel H. Wilson, Dante Marciani, John D. Minna

Cooperating Units: Laboratory of Cell Biology, NCI; Laboratory of Biochemical Genetics, NHLI

Man Years: 4
Professional: 3
Other: 1

Project Description:

Objectives: To study the synthesis and transport of individual macromolecules and their assembly into complex intracellular components; to study the effects of neoplastic transformation on these coordinated functions.

Methods Employed: Cell fractionation by differential and density gradient centrifugation, isolation and gradient fractionation of nucleic acids and nucleoproteins, electron microscopy of isolated nucleoproteins, in vivo and in vitro incorporation of isotopically-labelled precursors into protein and nucleic acid, chromatographic, immunochemical and electrophoretic characterization of specific proteins, tissue culture of normal and transformed cells.

Results: Studies on the chemistry and biological role of intracisternal A-particles were continued. These virus-like structures form by budding at the endoplasmic reticulum and resemble morphologically the immature forms of certain oncogenic RNA viruses. They occur very rarely in normal cells of adult mice. However, they appear with regularity (although sparsely) in mouse embryos, and are quite numerous in many types of primary and transplanted mouse tumors.

1. Intracisternal A-particles from widely different types of mouse tumors contained similar structural proteins and shared antigenic reactivity. The group specific A-particle antigen has thus far been identified in extracts of normal mouse embryo and in a total of 16 tumor lines in 6 mouse strains. We have found no apparent relationship either immunologically or in terms of the molecular weight distribution of their constituent proteins between intracisternal A-particles and the C- and B-type oncogenic viruses, nor between intracisternal A-particles and the intracytoplasmic A-particles associated with mouse mammary tumor agent.

2. The major structural proteins have been isolated from intracisternal A-particles of two mouse myelomas and a cultured line of neuroblastoma cells. Peptide mapping of the proteins is in progress to provide a rigorous comparison of particles produced by different tumor lines and to examine possible sequence homologics with the proteins of known oncogenic viruses.

3. Some RNA preparations from isolated A-particles have recently been found by gel electrophoresis to contain a discrete component with apparent molecular weight near 3×10^6. This is significantly larger than the normal cytoplasmic RNAs and could represent an A-particle-specific RNA.

4. Isolated A-particles were shown to contain an enzyme which promotes the synthesis specifically of poly (dT) on natural or synthetic RNA's containing poly (rA) sequences. These findings are described in detail in Annual Report NCI-5214, prepared by Dr. S. H. Wilson. In terms of template specificity, conditions for optimal activity and response to specific antiserum, this enzyme differs from the reverse transcriptases of murine leukemia and mammary tumor viruses.

5. A-particle specific antigen is being assayed in a number of hybrid cell lines derived from parent cells that differ widely in antigen content (mouse:mouse hybrids) or in which one parent is devoid of detectable antigen (rat:mouse and hamster:mouse hybrids). In neuroblastoma: L-cell hybrids (mouse:mouse), high A-particle antigen content has been dominant, and remarkable amplifications have occurred in some hybrid clones. In an interspecies hybrid, HTC (rat): L-cell, A-particle antigen production was strongly suppressed (although C-type particles were produced in abundance).

Significance to Cancer Research: Intracisternal A-particles may represent the expression of an integrated viral genome which is distinct from the recognized oncogenic RNA viruses but possibly as widespread in normal and neoplastic mouse cells. Particles of like morphology have been reported occasionally in tumor cells from guinea pig, rat, gerbil and man, raising the possibility that similar genetic information is contained in cells of these species. It is important to determine whether the A-particle genome is biologically significant in neoplastic transformation. This work supports Objective 3, Approach 2 of the National Cancer Plan.

Proposed Course of Project: Studies in the categories listed under Results will be continued. Particular emphasis will be placed on the appearance and biological roles of A-particle proteins and associated polymerase activity in embryonic tissues and in cells undergoing neoplastic transformation (e. g., methylcholanthrene-treated epidermis). Synthesis and assembly of the A-particles will be studied in established tissue-culture cell lines (myeloma and/or neuroblastoma).

Honors and Awards: Principal Investigator received PHS Meritorious Service Award.

Publications:

1. Kuff, E. L., Lueders, K. K., Ozer, H. L., and Wivel, N. A.: Some structural and antigenic properties of intracisternal A-particles occurring in mouse tumors. Proc. Nat. Acad. Sci. 69: 218-222, 1972.

Serial No. NCI-5210
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Control of gene expression and growth in animal cells

Previous Serial No.: Same

Principal Investigator: E. Brad Thompson

Other Investigators: E. L. Kuff, S. H. Wilson, J. V. Miller, Jr., and D. Aviv

Cooperating Units: Dr. Marc Lippman and Dr. Seymour Perry, Chemotherapy Branch, NCI; Dr. D. Nebert and W. Benedict, Laboratory of Biomedical Sciences, CH; Dr. F. K. Millar, Laboratory of Physiology, NCI; Dr. M. Feigelson, Dept. of Endocrinology, Columbia Univ. College of Physicians and Surgeons; Dr. S. S. Yang, Bionetics Research Labs.; Dr. S. R. Levisohn, Laboratory of Biochemical Genetics, NHLI; Dr. E. Henderson, B. Levinthal and R. Halterman, Medical Branch, NCI; Dr. V. Wyatt, Laboratory of Biology, NCI; and Dr. R. Kroes, Experimental Pathology Branch, NCI.

Man Years:
Total: 7
Professional: 5
Other: 2

Project Description:

Objectives: The prime objective is to learn as much as possible about the control of growth and gene activation. Additional goals are to determine the mechanism of action of steroid hormones and the correlation between steroid binding and response to steroid therapy in leukemia cells.

Methods Employed: The methods used are tissue culture, spectrophotometric, spectrophotofluorometric and radioactive enzyme assays, radioimmunoprecipitation, cell fusion, reverse-phase chromatography, radioautography, microscopy, affinity chromatography, cell-free protein-synthesizing systems,

chromosomal analysis, chemical mutagenesis, cell cloning, gradient centrifugation, etc.

Major Findings:

1) Cell fusion studies:

a) Hybrids between L-cells (tyrosine aminotransferase -) and HTC cells (tyrosine amiontransferase +) show contact inhibition of growth, whereas neither parent does so, both being transformed cell lines. The hybrids do not express the inducible HTC enzyme, tyrosine aminotransferase, but nevertheless seem to possess specific steroid binding proteins.

b) Hybrids between 3T3 cells (tyrosine aminotransferase - aryl hydrocarbon hydroxylase +) and HTC cells (tyrosine aminotransferase +, aryl hydrocarbon hydroxylase -) are - for tyrosine aminotransferase, but show enhanced levels of inducible aryl hydroxylase. These hybrids seem to lack steroid binding protein.

c) Short-term HTC x BRL62 (tyrosine transaminase -) heterokaryons are histochemically negative for the enzyme.

d) Attempts are being made to fuse normal liver cells with a variety of other cell lines.

2) Cell-free protein synthesis:

a) Initiation of globin synthesis has been acheived in cell-free conditions using preparations from HTC or mouse ascites cells. A repressive effect on such synthesis has been found to be exerted by polymers of desoxythymidylic acid. Polyriboadenylic acid counteracts this effect.

b) Using ribosomes enriched for tyrosine aminotransferase synthesis by the affinity chromatography method we developed last year, we found no control exerted on the extension of enzyme chains in the process of synthesis by extracts of cells not induced for the enzyme or of cells totally lacking the enzyme.

3) Induction of phe tRNA by steroids in vivo which we have previously discovered has been confirmed under cell-free conditions, and reverse-phase chromatography has shown the increase to be confined to one of the two phe tRNA isoacceptors.

4) Our histochemical method for demonstrating tyrosine aminotransferase in tissue culture has been adapted for use with quick-frozen animal tissues.

5) Cellular genetic studies:

a) Further analysis of transaminase phenotypes in HTC subclones has shown that this enzyme is expressed at a rate of variability too great to explain by simple random mutagenesis occurring at the usual rates.

b) Additional amino-acid prototrophic lines have been derived from HTC wild-type.

6) Steroid binding studies have been begun in mouse and human leukemic cells Search for specific steroid binding sites seems to show a heterogeneity of these in various forms of leukemia. We intend to try to define this hetero-

geneity, to correlate steroid binding and/or its absence with resistance to steroid therapy, and to use these data to try to develop more rational anti-leukemic therapy.

7) Affinity chromatography has been used to purify tyrosine aminotransferase.
8) A radiochemical assay for tyrosine aminotransferase has been developed.

Significance to Cancer Research: We want to know how animal genes are controlled. It is our belief that cancer is, in some way, a disfunction of normal controls over gene expression. If for even a single animal gene, the exact controlling mechanisms were known, it would provide a much more powerful way to consider cancer and its control than any we now have. Most of the above findings obviously are aimed to this goal at one level or another. In addition, we are applying when it is possible to do so, the special knowledge and techniques our basic studies have given us, to try to improve rational and practical cancer therapy (cf. item 6 above). Objective 2, approach 3.

Proposed Course of Research: Cell hybridization will continue to be used to study control functions in gene expression. Emphasis will be placed on the use of short-term heterokaryons to avoid ambiguities introduced by selective chromosome losses.

The interesting HTCxL cell hybridization will be repeated to see if the alteration from non-regulated growth to regulated growth occurs. We are discussing the possibility of achieving such regulation by introducing chromosomes into transformed cells, in collaboration with Dr. McBride.

Cell-free amino acid incorporation studies will be continued to investigate the regulation of tyrosine transaminase formation as well as factors that may be more generally important in controlling rate and specificity of protein synthesis.

Steroid-binding properties of leukemic and tissue culture cells will be investigated. An effort will be made to correlate the level of steroid-binding proteins in human leukemic cells with the clinical response to steroid therapy, in order to develop a predictive criterion for such treatment.

Honors and Awards: None

Publications:

1. Thompson, E. B., and Gelehrter, T. D.: Expression of tyrosine aminotransferase activity in somatic-cell heterokaryons: Evidence for negative control of enzyme expression. Proc. Nat. Acad. Sci. USA 68: 2589-2593, 1971.

2. Levisohn, S. R., and Thompson, E. B.: A variant in the regulation of tyrosine aminotransferase induction in tissue culture. Nature-New Biology 235: 102-104, 1972.

3. Miller, J. V. Jr., and Thompson, E. B.: A new radioactive assay for tyrosine aminotransferase. Anal. Biochem. In press.

4. Benedict, W., Nebert, D., and Thompson, E. B.: Expression of aryl hydrocarbon hydroxylase indiction and supression of tyrosine aminotransferase induction in somatic-cell hybrids. Proc. Nat. Acad. Sci. USA. In press.

Serial No. NCI-379
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Ultrastructural characterization of immunoglobulins, enzymes and other biomacromolecules

Previous Serial No.: Same

Principal Investigator: Emma Shelton

Other Investigators: R. M. Stroud and K. Yonemasu

Cooperating Units: Division of Clinical Immunology and Rheumatology, Department of Medicine, University of Alabama Medical Center, Birmingham, Alabama

Man Years:
Total: 3
Professional: 2
Other: 1

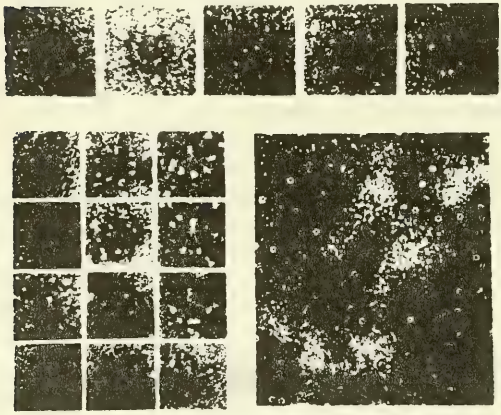
Project Description:

Objectives: To relate the fine structure of macromolecules to their biochemical and physical properties, and to their function in biological systems.

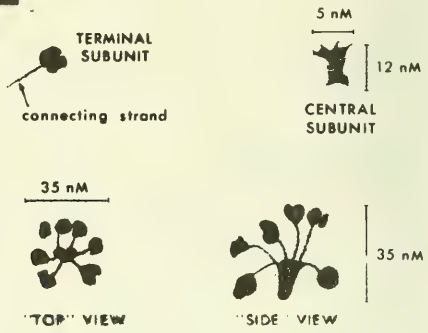
Major Findings:
Solution of the ultrastructure of the human complement component, C1q. C1q is the largest of the complement proteins (M. W. 400,000; 11.1S) and since the molecule occupies the key position as the protein that activates the whole complement system, a knowledge of its ultrastructure may provide clues as to how it functions. During the past year, in collaboration with R. M. Stroud and K. Yonemasu at the University of Alabama Medical Center, we have been able to resolve the structure of this important protein.
Purification, electron microscopy: The molecule was extremely labile to heat, and storage, thus, it was essential to examine the biologically active preparations immediately upon isolation from freshly drawn human blood. The isolation procedure relied upon the differential precipitation of C1q from serum at low ionic strength and its dissociation from C1r and C1s in the absence of Ca^{++} ions. The purified, diluted protein was negatively stained

with uranyl acetate; examined and photographed with a Siemens 101 electron microscope.

Structure: The Clq molecule consists of three basic parts, a) terminal subunits, b) connecting strands, and c) a central subunit. Each terminal subunit is further divided into a large and a small subunit; morphologic evidence suggests that the central subunit also is divided longitudinally into two parts. In the intact molecule, six connecting strands join the terminal subunits by their larger subdivision to the central subunit. Photographs and models of the molecule appear below.



STYLIZED ULTRASTRUCTURE OF Clq MOLECULE



Conclusions: The shape of Clq is similar to the immunoglobulin, IgM, in that its shape may reflect its function; both molecules have the possibility for flexibility built into their structure and both molecules act as physical links in the interacting molecular systems of which they are a part.

Significance to Cancer Research: A knowledge of the structure of macro-

molecules obtained by observations with the electron microscope provides in many cases an unequivocal basis for a knowledge of their function as well as an unequivocal confirmation of physical-chemical data. Thus, we have shown that the complement component, Clq, has a flexible structure that would permit conformational change upon interaction with antigen-antibody complexes.

Proposed Course of Research: To elucidate further the structure of immunoglobulins and complement by examination of the fine structure of their subunits. To study conformational changes in (a) immunoglobulins upon interaction with antigen and (b) complement upon interaction with immunoglobulins.

Honors and Awards: Invited speaker, workshop on ultrastructure of immunoglobulin and Ag-Ab complexes. First International Congress of Immunology, Washington, D. C.

Publications:

1. Shelton, E., and Smith, M.: The ultrastructure of Carp (Cyprinus carpio) immunoglobulin: A tetrameric macroglobulin. J. Molec. Biol. 54: 615-617, 1971.

2. Acton, R. T., Weinheimer, P. F., Hall, S. J., Niedermeier, W., Shelton, E., and Bennett, J. C.: Tetrameric immune macroglobulins of three orders of bony fishes. Proc. Nat. Acad. Sci. USA 68: 107-111, 1971.

3. Johnson, W. H. Jr., Acton, R. T., Weinheimer, P. F., Niedermeier, W., Evans, E. E., Shelton, E., and Bennett, J. C.: Isolation and physico-chemical characterization of the "IgM-like" immunoglobulin from the Stingray Dasyatis americana. J. Immunol. 107: 782-793, 1971.

Serial No. NCI-5215
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biological and ultrastructural studies on cell cooperation in the immune response

Previous Serial No.: Same

Principal Investigator: Emma Shelton

Other Investigators: W. L Bennett and M. G. Mage

Cooperating Units: Section on Biophysical Histology, Laboratory of Experimental Pathology, NIAMD

Man Years:
Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: To examine the interaction of lymphocytes and macrophages cooperating in the immune response in order to answer questions relating to:
a) localization of antigen and antibody in cooperating cells.
b) the role of the macrophage in the immune response.
c) the types of lymphocytes (thymus-dependent or bone marrow-dependent) responding to various antigens.

Major Findings:
1) Setting-up a model system which will permit biological and ultrastructural examination of cell cooperation in the immune response. The diffusion chamber constructed with cellulose membrane filters and implanted by laparotomy in mice represents a culture system that permits long-term cultur of macrophages and lymphocytes (Shelton and Rice, Amer. J. Anat. 105: 281-341, 1959). Cells growing in the chamber can readily be labeled by intraperitioneal injection of radioisotopes and can be treated as a tissue for examination by light or electron microscopy.
2) Experiments with sheep erythrocytes as antigen. Cells washed from the

peritoneal cavity of mice previously immunized with 2 x 10^8 sheep erythrocytes will produce hemagglutinating antibody when cultured in diffusion chambers with equal numbers of sheep erythrocytes (see annual report, 1970-71). These results have satisfied us that mouse peritoneal cells (lymphocytes, 60%, macrophages, 40%) have the capacity to produce high antibody titers in the diffusion chamber system. A result of interest from these studies was the persistence of large numbers of plasma cells and high antibody titers (4-12 logs) after 67 days in culture. This suggests that antibody-producing plasma cells either have a longer life than hitherto suspected or that the plasma cell has the capacity to continue to reproduce functioning progency for long periods of time.

3) Experiments with horseradish peroxidase (HRP) as antigen. HRP is a small molecule (40,000 M. W.) that has been used extensively as a tracer in immunological and physiological experiments at the electron microscope level. The enzyme reacts with the substrate, 3,3'-diaminobenzidine to produce a product that is blackened by osmium tetroxide and rendered visible in the electron microscope. Following glutaraldehyde fixation and incubation in the presence of HRP, cells producing anti-HRP antibodies can be identified. HRP couples specifically to antibody present in the cisternae of the rough endoplasmic reticulum and Golgi apparatus and the black product can be seen in these regions following the reaction with diaminobenzidine.

We are using HRP to trace the cellular events leading to antibody production in the secondary response using "immune" peritoneal cells grown in diffusion chambers.

Results with this system were as follows: Immunization of mice: Mice (strain AAF_1 females) immunized by subcutaneous injection of 250-500 μg of HRP in complete Freund's adjuvant begin to produce circulating antibody (both precipitating and agglutinating) in about 30 days and continue to sustain reasonable titers for several months. These mice provided the "immune" cells for the diffusion chamber experiments. Production of antibody by "immune" cells in diffusion chambers: We have succeeded in demonstrating intracellular anti-HRP antibody in plasma cells in an 8-day old culture of peritoneal cells from an immune animal that were exposed to HRP before culture. Although we are having trouble repeating this experiment (the size of the HRP molecule makes it an unreliable antigen) we are greatly encouraged with this results since it has shown that technically we now have succeeded in performing every step toward initiating and visualizing antibody production in the diffusion chamber.

Retention of antigen on cell surfaces: Knowledge of the fate of antigen when exposed to macrophages and lymphocytes is essential to an understanding of the inductive phase of the immune response. Experiments are in progress to visualize HRP retained on the surface of macrophages and lymphocytes with anti-HRP antibody coupled to ferritin. A carbon replica of the surface of these cells will be examined in the electron microscope after shadowing with

a heavy metal such as platinum. The distribution of the ferritin label over the cell surface may provide clues as to the relationship between portions of the membrane retaining antigen and portions of the membrane active in phagocytosis-pinocytosis.

Preparation of ferritin-coupled antibody: Results of preliminary experiments with sheep antibody to rabbit gamma globulin; 1) Purification of specific antibody from non-specific immunoglobulin proved to be an essential step in the labeling procedure since specific ferritin-labeled antibody could not be detected by immunodiffusion or immunoelectrophoresis in the presence of large amounts of non-specific antibody. 2) Ferritin coupling: Successful conjugation of ferritin to purified sheep immunoglobulin (supplied by M. Mage) was accomplished by a standard method using toluene-2, 4-diisocyate as the coupling agent. 3) Separation of the conjugate from unconjugated antibody was accomplished by ultracentrifugation and by gel filtration. The latter method reduced contamination of conjugate with uncoupled ferritin and will be the method of choice in the future.

The purification of rabbit anti-HRP antibody from the gamma globulin fraction of serum from rabbits hyperimmunized to HRP has been accomplished by affinity chromatography on a column of cyanogen bromide-activated agarose beads coupled directly to HRP or indirectly to HRP-hexane diamine. When available in sufficient quantity, this material will be coupled to ferritin and used as the electron dense label to visualize HRP on cell surfaces.

Significance to Cancer Research: An examination at the ultrastructural level of cells interacting in the immune response should lead to an expansion of our knowledge of how such cooperation is effected. An understanding of such cooperation has ultimate usefulness in the treatment of cancer and other diseases. Objective 3, Approach 4 of the National Cancer Plan.

Proposed Course of Research: By using tracers visible at the ultrastructural level, we hope to follow the interaction of macrophages and lymphocytes during the production of antibody in the secondary response of these cells while growing in diffusion chambers. By separating and recombining macrophages and lymphocytes, we hope to visualize ways in which the macrophage participates in the immune response.

Honors and Awards: Associate Editor, Cancer Research

Publications: Not yet

Serial No. NCI-5212
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Virus-cell interaction of oncogenic viruses: Selection of temperature sensitive mutants of BALB/3T3

Previous Serial No.: Same

Principal Investigator: Harvey L. Ozer (now of Worcester Foundation for Experimental Biology, Shrewsbury, Mass.)

Other Investigators: Robert E. Wittes, Carmen Upenieks

Man Years:
Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: To study the interaction of oncogenic viruses, particularly SV40, with mammalian cells, utilizing temperature-sensitive mutants of BALB/3T3.

Methods Employed: Routine tissue culture and virological techniques. Chemical mutagenesis of cells. Routine methods of labeling cellular macromolecules with radioactive small molecules.

Major Findings: In the course of the past 2 years attempts have been made to isolate temperature-sensitive mutants of BALB/3T3 specifically defective in DNA synthesis. BALB/3T3 was chosen because of the ability of this cell line to support replication by polyoma and transformation by SV40, as well as infection by the RNA tumor viruses. With such mutant studies of the relationship of the various host functions important in DNA synthesis to viral transformation could be undertaken. To date, only one temperature-sensitive mutant of BALB/3T3 has been isolated. The specific defect in this cell line has not yet been clarified but experiments thus far show no evidence for preferential early cessation of DNA synthesis. The cell does exhibit several interesting features, among which is an apparent density-dependence of the phenotype; that is, the cell shows temperature sensitivity

of growth when plated at low cell densities, but this property is largely reversed when the cells are plated at higher densities. Experiments to explore this property, as well as to investigate the host properties of this cell line with respect to polyoma and SV40 are in progress.

Studies described last year on the synthesis and assembly of SV40 protein components have been published. This work ceased with the departure of the principal investigator in January, 1972.

Significance to Cancer Research: Availability of a series of temperature-sensitive mutants defective in DNA synthesis would be of great assistance in the attempt to contributions of host cell and virus in the transformation process, since such mutants would allow the identification of specific host functions necessary for viral transformation. National Cancer Program: objective 3, approach 1 and 2.

Proposed Course of Research:

1) Continue characterization of the mutant line already isolated.
2) Continue attempts to select further mutant lines (by Dr. Ozer at Worcester Foundation).

Honors and Awards: None

Publications:

1. Tegtmeyer, P. and Ozer, H. L.: Temperature-sensitive mutants of SV40: Infection of permissive cells. J. Virology 8: 516-524, 1971.

2. Ozer, H. L.: Synthesis and assembly of SV40. I. Differential synthesis of intact virions and empty shells. J. Virology 9: 41-51, 1972.

3. Ozer, H. L., and Tegtmeyer, P.: Synthesis and assembly of SV40. II. Synthesis of the major capsid protein and its incorporation into viral particles. J. Virology 9: 52-60, 1972.

Serial No. NCI-5218
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The thymidine triphosphate pool in L-cells

Previous Serial No.: None

Principal Investigator: Robert E. Wittes

Other Investigators: William R. Kidwell

Cooperating Units: None

Man Years:
Total: 2
Professional: 2
Other: 0

Project Description:

Objectives: To study the TTP pool size in growing cultures of mouse L-cells and specifically to compare the size of the pool as determined kinetically with that determined chemically.

Method Employed: Routine tissue culture techniques and routine methods of labeling cells with radioisotopes. Enzymatic assays of intracellular TTP.

Major Findings: Previous studies of nucleoside triphosphate pool sizes in mammalian cells in culture have relied on direct determination of the desired compound by one or another chemical method . None of the methods to date have been capable of determining whether there is more than one pool of intracellular nucleotide. To this end, a simple mathematical model has been developed which relates several parameters, among which is the intracellular TTP pool size to the kinetics of incorporation of radio-labeled thymidine into the DNA of exponentially growing cultures of L-cells. Labeling experiments have been carried out in order to provide data by which the pool size may be determined. For comparison with the kinetically determined pool size, the pool size will also be determined directly by standard methods. Comparison of the values obtained by these two methods will allow one to say whether the entire TTP pool in the cell is being used for DNA synthesis.

Significance to Cancer Research: Much work on the mechanism of certain anti-tumor agents, notably amethopterin and cytosine anabinoside, has focused on the apparent effect of these drugs on the size of intracellular pools of nucleoside triphosphates. It is obviously of importance to be sure that the pool one determines by the usual methods is in fact the pool which provides the precursors for DNA synthesis. National Cancer Program: objective 3, approach 1.

Proposed Course of Research: This work will terminate with the departure of the principal investigator from NIH in July 1972.

Honors and Awards: None

Publications: None

Serial No. NCI-5214
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Regulation of nucleic acid metabolism

Previous Serial No.: None

Principal Investigator: Samuel H. Wilson

Other Investigators: E. L. Kuff, J. V. Miller, Jr. and E. B. Thompson

Cooperating Units: None

Man Years:
Total: 1 3/4
Professional: 1
Other: 3/4

Project Description:

Objectives: The broad objective of the research program is to understand mechanisms of regulation of nucleic acid metabolism in eucaryotic cells.

During the current year the aim has been to investigate DNA polymerase activity found in association with intracisternal A-type particles from mouse tumors and to determine the effect of this enzyme(s) and its product on cellular and A-type particle biology.

Methods Employed: In order to quantitatively measure low levels of DNA polymerase activity in crude cell extracts, a radiochemical assay was developed in which unstable enzyme activities are protected and where nucleotide substrates are continuously generated during the course of reactions. Cell fractionation and assay of reaction products are prepared by conventional techniques of density equilibrium centrifugation, scintillation spectrophotometry, and column chromatography.

Major Findings:
1) Detection of DNA polymerase activity in preparations of intracisternal A-type particles from mouse tumors. A DNA polymerase activity detected in preparations of intracisternal A-type particles appears to be a new template specific RNA-directed DNA polymerase ("reverse transcriptase").

The enzyme activity promotes synthesis of poly(dT) only, in the presence of RNA templates containing poly(rA). Some properties of the enzyme activity including high salt requirement, divalent cation preference, and activity in the presence of neutralizing antiserum against C-type particle "reverse transcriptase" appear to distinguish the A-type particle DNA polymerase from other known particle or viral associated DNA polymerase activities.

2) The DNA polymerase activity appears to be firmly associated with intracisternal A-type particles. Virtually all of the poly(rA)-directed poly (dT) polymerase activity in a crude cytoplasmic membrane fraction containing A-type particles was found to sediment with A-type particles upon density equilibrium centrifugation in sucrose gradients. The activity remained particle associated after a second centrifugation to equilibrium and 60% of the activity remained particle associated after washing in 0.5 M KCl. The DNA polymerase activity associated with A-type particles has not yet been detected in cells that do not contain A-particles or in subcellular fractions that are particle free.

3) The A-type particle DNA polymerase activity is latent in crude(mitochondrial; microscomal)membrane fractions and may be activated by surfactants. Detection of the poly(rA)-directed poly(dT) polymerase activity in crude membrane fractions (known to contain A-type particles) was found to be dependent upon the presence of surfactants in the reaction mixture.

A variety of surfactants have been tested for their ability to activate the latent enzyme activity. With use of surfactant activation and specific assay conditions it will be possible to determine the intracellular distribution of the new DNA polymerase activity and its level of activity in crude extract from a variety of tissues.

4) Poly(dT) is an inhibitor of rabbit globin messenger RNA translation in a cell-free system. A possible role of a poly(rA) $\rightleftarrows$ poly(dT) conversion mechanism has been studied by determining the effect of poly(dT) upon translation of messenger RNA containing poly(rA) sequences. The in vitro production of globin by direction of rabbit reticulocyte 9S RNA in a Krebs II ascite cell system was inhibited competitively by poly(dT). The poly(dT) inhibition was partially reversed by poly(dA) and was detected when poly (dT) was substituted with oligo(dT).

Significance to Cancer Research: A detailed understanding of the mechanism and regulation of DNA synthesis is one of the most important unsolved problems in the study of the molecular biology of Cancer. Advances on all levels in our understanding of DNA synthesis should prove useful in preventing, treating and ultimately controlling Cancer.

RNA-directed DNA polymerase activities have recently been implicated in the biology of oncogenic RNA viruses; the RNA-directed DNA polymerase under study in this project also may be an important agent in the biology of tumors and some rapidly growing eucaryotic cells. National Cancer Program;

objective 3, approach 1 and 2.

Proposed Course of Research:
1) To further purify and characterize the A-type particle DNA polymerase.
2) To determine the intracellular distribution and regulation of the DNA polymerase activity found in association with A-type particles and to determine whether it occurs in various tissues other than A-particle containing mouse tumors.
3) To investigate the possible role of this and other RNA-directed DNA polymerases on cellular metabolism.

Honors and Awards: None

Publications:

1. Wilson, S. H., and Kuff, E. L.: A novel DNA polymerase activity found in association with intracisternal A-type particles. Proc. Nat. Acad. Sci. USA. In press.

Serial No. NCI-5217
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of mechanisms of information transfer between cells in the mammalian nervous system

Previous Serial No.: None

Principal Investigator: Samuel H. Wilson

Other Investigators: M. W. Nirenberg, B. K. Schrier, E. J. Thompson, and R. Pertel

Cooperating Units: Laboratory of Biochemical Genetics, National Heart and Lung Institute, Biomedical Eng. & Inst. Br. DRS, NIH

Man Years:
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: To develop techniques and procedures for investigation of communication between cells in the nervous system.

Methods Employed: Culture of primary cells and established cell lines was performed in monolayer on surfaces of plastic and collagen. Movement of cells in culture was assessed by television video analysis. Enzyme activities were measured using radiometric assays and radioactive reaction products were assessed by high voltage electrophoresis, ion-exchange column chromatography and thin-layer chromatography.

Major Findings: New methods were developed for (1) the culture of cells from the mouse nervous system; (2) preparation of cell homogenates and (3) quantitative radiochemical determination of histamine content and activities of choline acetyltransferase, tyrosine hydroxylase, catechol o-methyltransferase, glutamate decarboxylase and acetylcholinesterase. In addition two new techniques were developed for the measurement of the rate and velocity of movement by single muscle cells in culture.

Significance to Cancer Research: This research project has produced a number of new techniques for study of gene expression in mammalian cells and thus has made an important contribution to basic cell biology. Ultimately, a more thorough understanding of gene expression in mammalian cells will provide guides to control of malignant growth and thus will provide new insight into practical cancer therapy and prevention. This work if most closely related to objective 3, approach 2 of the National Cancer Plan.

Proposed Course of Research: No further work in this area is anticipated.

Honors and Awards: None

Publications:

1. Wilson, S. H., Schrier, B. K., Farber, J. L., Thompson, E. J., Rosenberg, R. N., Blume, A. J., and Nirenberg, M. W.: Markers for gene expression in cultured cells from the nervous system. J. Biol. Chem. In press.

2. Thompson, E. J., Wilson, S. H., Schuette, W. H., Whitehouse, W. C., and Nirenberg, M. W.: Measurement of the rate and velocity of movement by single heart cells in culture. Amer. J. Card. In press.

3. Wilson, S. H., Schrier, B. K., and Nirenberg, M. W.: Assay of enzymes of neurotransmitter metabolism from neuroblastoma and other cultured cells from the nervous system. In Fleischer, S., Packer, L., and Estabrook, R. W. (Eds.): Methods in Enzymology. New York, Academic Press, In press.

4. Hamprecht, B., Schrier, B. K., Wilson, S. H., Amano, T., and Nirenberg, M. W.: Assay for choline acetyltransferase. In Fleischer, S., Packer, L., and Estabrook, R. W. (Eds.): Methods in Emzymology. New York, Academic Press, In press.

Serial No. NCI-368
1. Laboratory of Biochemistry
2. Tumor Physiopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the mechanisms of mammary tumor regression

Previous Serial No.: Same

Principal Investigator: Pietro M. Gullino

Other Investigators: F. H. Grantham, B. Berghoffer, and I. Losonczy

Cooperating Units: None

Man Years:
Total: 4
Professional: 1
Other: 3

Project Description:

Objective: To understand the mechanism of regression in hormone responsive mammary tumors.

Methods Employed: Transplantable MTW9 and primary 7-12 DMBA induced mammary tumors are used as models. Primary, methyl nitroso urea or methylcholanthrene induced mammary tumors of BUF/N ♀ rats are being tested for possible use in this study. Tumor regression is induced by castration and/or removal of pituitary hormones. Procedures developed in previous years, such as the tissue isolated tumor preparations or sampling of tumor interstitial fluid by micropore chambers are being applied to the study of tumor regression. A new procedure to study in vivo physiopathological parameters of primary mammary tumors has been developed (See results).

Major Findings:
1) A method for obtaining a primary mammary tumor connected to the host by a single artery and vein has been successfully developed. This preparation permits 2 experimental approaches: a) collection of the whole blood flowing out of the primary tumor, and b) preparation of a primary tumor detached from the host as an isolated organ. Use of this preparation in the

study of cell loss by the primary tumor is being planned.
2) A procedure for mammary tumor carcinogenesis by methyl nitroso urea (MNU) has been developed and a contract for routine production of these tumors is operative. The purpose is to have a primary mammary tumor model more "malignant" than 7-12 DMBA tumors and metastasizing. MNU induces tumors which satisfy the first but not the second requirement. Attempts to induce distant metastases in this model system are going on.
3) The site of lysosomal enzyme action during regression has been defined. The activity of 6 lysosomal enzymes was shown to be increased during regression in the whole tumor homogenate but not in the fluid surrounding the regressing cells in vivo. Lysosomal enzymes were placed in the interstitial fluid of the tumor in vivo and were shown to be active. Inactivity during regression was, therefore, interpreted as due to absence of the enzymes in the interstitial fluid. Regression appears to be an autophagic process limited to and probably regulated by each cell. Since hormonal deprivation involves the whole tumor but regression does not hit all cells simultaneously, there is probably a cell dependent trigger which initiates autophagy. Efforts to increase our understanding in this area are going on.
4) A burst of RNA-labelling (using ^{3}H-uridine as precursor occurs in the regressing tumor within a few hours after hormonal deprivation of the host, followed by incorporation of C^{14} (using glycine as precursors) into protein and lipids. New molecules of lysosomal enzymes are formed at this time (see project no. 5216). Label incorporation into RNA is mostly limited to the nucleus. Pulse label and chase experiments indicate that within 12 hours labelled RNA has passed into the cytoplasm. A sharp increment of (label incorporation into) RNA labelling in regressing tumors is produced by estradiol 17β with a peak at 20 minutes after injection, followed by increased incorporation of label in proteins and lipids. The events occurring during growth resumption of a regressing tumor stimulated by estradiol 17β appear to follow the same pattern as the estrogen stimulated growth of atrophic uterus.
5) Actinomycin D given to the host blocks the increment of label incorporation into RNA of the tumor but does not arrest regression. Puromycin and cycloheximide block the increment of protein synthesis which follows label incorporation into RNA at the onset of regression but they do not block regression. Cycloheximide blocks also label incorporation into RNA, suggesting that new formation of protein(s) precedes the burst of RNA synthesis observed at the onset of regression. The inability of arresting regression (besides hormonal stimulation which induces growth) is the most severe factor limiting our efforts in this area.

Significance to Cancer Research: The work contributes to the understanding of growth arrest and regression of experimental mammary tumors. Since the experimental models mimic the behavior of human breast cancer, this

work contributes to the understanding of the human disease. National Cancer Program: objective 4, approach 1 and 2.

Proposed Course of Research: To continue the study of the mechanism of mammary tumor regression.

Honors and Awards: None

Publications:

1. Watters, C., and Gullino, P. M.: Translocation of DNA from the vascular into the nuclear compartment of solid mammary tumor. Cancer Res. 31: 1231-1243, 1971.

Serial No. NCI-5216
1. Laboratory of Biochemistry
2. Tumor-Physiopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Role of lysosomal enzymes in the regression process of mammary tumors

Previous Serial No.: Same

Principal Investigators: Yoon Sang Cho-Chung and Richard H. Lanzerotti

Other Investigators: Pietro M. Gullino

Cooperating Units: None

Man Years:
Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: This project has been concerned with the role of lysosomal enzymes in the regression of hormone-dependent rat mammary carcinomas. Specifically, our objectives have included: 1) Determination of the sequence and magnitude of change of several acid hydrolases during tumor regression; 2) Correlation of changes in enzyme activities with changes in enzyme quantities; 3) Determination of whether de novo synthesis of lysosomal enzymes occurs during regression; and 4) Evaluation of the relative rates of enzyme synthesis and degradation in regressing tumors compared to growing ones.

Methods Employed:
1) Two hormone-dependent rat mammary carcinomas were used in our studies: a) a primary tumor, 7-12 DMBA, regresses following surgical castration of the host, b) a transplantable tumor, MTW9, regresses following surgical removal of a mammotrophin-producing pituitary tumor and host castration.
2) Monospecific antisera against acid ribonuclease and β-glucuronidase were produced in rabbits after the enzymes were purified by standard techniques.

3) A multispecific antiserum was produced against a lysosome-enriched fraction from the MTW9 mammary tumor; it contained antibodies to acid phosphatase, β-galactosidase, and β-glucuronidase and two other antigens.
4) The single radial immunodiffusion technique, with a histochemical modification, was used with both the monospecific and multispecific antisera to measure enzyme quantities.
5) Techniques of in vivo infusion of radioactive precursors were used to label tumor protein, and immunochemical techniques were used to precipitate specific enzymes.

Major Findings: Our investigations have yielded the following findings:
1) Lysosomal enzymes significantly increase in total and specific activities during early stages of hormone-dependent mammary tumor regression. The sequence of change in enzyme activities and the magnitude of change varies from one enzyme to another. Acid ribonuclease increases the earliest (6 hours following hormone deprivation), and β-glucuronidase increases the most in activity (350% increase in specific activity compared to growing tumor activity).
2) Regressing tumors contain significantly greater quantities of enzyme than do growing tumors, and the changes in quantities of acid phosphatase, β-galactosidase, and β-glucuronidase appear to be similar to changes in activities.
3) Changes in the amount of acid phosphatase, β-galactosidase, and β-glucuronidase could be accurately quantitated by using the multispecific antiserum with the single radial immunodiffusion technique. Several precipitin rings resulted from radial diffusion of crude supernatant fractions into the antiserum-agarose gels, but individual antibody-enzyme precipitin rings could be recognized by specific histochemical reactions.
4) Acid ribonuclease and β-glucuronidase are synthesized de novo in regressing tumors.

Significance to Cancer Research: The findings support and extent our previous ones. They indicate that de novo synthesis of lysosomal enzymes occurs during hormone-dependent mammary tumor regression. Because of the close similarity of the animal tumor models to the hormone-responsive human breast carcinomas, these findings contribute to the understanding of the induction of tumor regression. National Cancer Program: objective 4, approach 1 and 2.

Proposed Course of Research: We intend to continue the study of tumor regression and in particular the study of the role of degradative enzymes in the regression process.

Honors and Awards: None

Publications: None

Serial No. NCI-5219
1. Laboratory of Biochemistry
2. Tumor-Physiopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: In vitro culture of solid tumors

Previous Serial No.: None

Principal Investigator: Richard A. Knazek

Other Investigators: Pietro M. Gullino

Cooperating Units: BEIB; Endocrine Division of NICHD

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: Development of a technique to culture solid tumors in vitro with subsequent physiopathologic study.

Methods Employed: Cells were placed on one side of sterile tubular membranes while the other side was continually perfused by a standard cell culture medium. Periodic replacement of the medium prevented depletion of necessary nutrients or accumulation of toxic by-products from cellular metabolism.

Major Findings: L-cells which had been reported to grow only in monolayers, formed nodules 1-2 mm in diameter implying that a release from contact inhibition may be obtainable by this technique.

An established human choriocarcinoma cell line grew to multilayer depths.

Significance to Cancer Research: Cell densities may now be attained in vitro that approach those of solid organs. These organoid structures will undoubtedly behave differently from the same cells grown in monolayer forms and more likely possess the behavioral potential of the in vivo state. This will permit a more accurate assessement of tumor physiology under varying

conditions and a more correct evaluation of their response to drugs without introducing the variabilities inherent in in vivo studies. The possibility also exists that tumor cells which had previously defied standard culture techniques may now be cultivated and studied in vitro. National Cancer Program: objective 4, approach 1 and 2.

Proposed Course of Research: Application of this technique to other cell types will follow, the most pertient being the study of the growth and regression of hormonally responsive mammary carcinoma.

Honors and Awards: None

Publications: None

Serial No. NCI-334
1. Laboratory of Biochemistry
2. Cytochemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The occurrence and function of a stress-modifiable insulin: anti-insulin hormonal complex regulating glucolysis in cancer and normal cells, and its relation to chemotherapy at normal, and hyperthermic temperatures.

Previous Serial No.: Same

Principal Investigators: Mark W. Woods and Dean Burk

Other Investigators: None

Cooperating Units: Mr. George Vlahakis, Laboratory of Biology, GL and C, NCI; Dr. Katherine K. Sanford, Tissue Culture Section, Laboratory of Biology, GL and C, NCI; Dr. Walter Heston, Chief, Laboratory of Biology, GL and C, NCI.

Man Years:
Total: 3 3/4
Professional: 3
Other: 3/4

Project Description:

Objectives: To determine the extent to which regulation (e.g., insulin:anti-insulin) of glucolysis functions as a growth and cell function control system in different cancers and normal cells, and to find ways in which such systems can be manipulated as an adjunct to in vivo therapies (e.g., chemotherapy and stimulation of host defensive cells (RES). This project most closely supports the National Cancer Plan in objective 6 - Therapy (i.e., "to maintain maximum control of the cancerous process in patients not cured" through modification of control mechanisms of cell metabolism. The approach is 10.

Methods Employed: The glycolytic properties of tumor and normal tissues at various glucose levels are determined in the presence and absence of inhibitors such as 2-deoxy glucose, steroids, compounds with steroid-like anti-

glucolytic action such as delta[1]-testololactone, diethylstilbestrol, Podophyllum derivatives, and substances which counteract such glucolytic inhibition (e. g., insulin and bacterial endotoxins). We used these techniques in further studies of the glycolytic properties of a large number of primary and transplanted liver tumors and primary and transplanted mammary tumors of mice (Heston-Vlahakis strains of C_3H, C_3HfB, $C_3HA^{vy}fB$ and mammary tumors in $C_{57}BL$ and BALB/c mice). Certain other tumors were also used for comparison.

Major Findings: The following studies were carried out during 1971-72:
1) The stabilities of the glycolytic properties of transplanted liver tumors (e. g., Vlahakis hepatomas 12747, 16832, 16824, 18303, 16650 and cholangioma No. 1) were determined by standardized analysis of consecutive transplant generations (ca 3 months to a year apart) throughout the year. With the partial exception of 18303 and 16650 hepatomas (see below) the glycolytic patterns have remained remarkably constant during a year or more of transplantation rather strongly resembling patterns encountered in primary hepatomas in livers of the same mouse strains.
2) Continued glycolytic analyses of selected transplantable liver tumors under various conditions of in vitro incubation (e. g., with various glucose levels, glycolytic inhibitors, insulins, etc.). These studies also included comparisons with various non-hepatic tumors such as S91 melanoma, Ehrlich ascites carcinoma, mammary tumors etc.
3) Compared glycolytic properties of selected transplantable hepatomas grown in host strains of origin as well as in hosts of different strain and/or sex. So far the results have not indicated any marked effects of host strain with the possible exception of hepatoma 18303 growing in male $C_3HA^{vy}fB$ mice(cf item 4 next).Hepatoma 18303 originated in a male C_3H mouse over a year ago.
4) Morphologic and glycolytic studies showed the presence of at least two types of tumor cells in hepatomas 18303 and 16650. In 18303 a "sarcoma-like" component (hepatic cell derivative?) of high glucolytic capacity (low Km for glucose, high $Q_{CO_2}^{N_2}$) has been identified and is under study in various host strains. Data so far obtained suggest that in male $C_3HA^{vy}fB$ mice the "sarcoma-like" component may grow relatively faster than the ordinary hepatoma component. On the other hand, in C_3H males (or C_3HfB) it appears that the two types of tumor cells remain remarkably balanced in the tumor complex. In tumor 16650 an apparent invasion (?) by a rather hepatotrophic reticulum cell neoplasm (RCN) occurred and was detected morphologically and glycolytically in certain transfer lines of the hepatoma. Host liver-RCN and hepatoma-RCN mixtures, as expected, displayed mixed glycolytic profiles.
5) A strongly and selectively hepatotrophic RCN tumor (discovered by Mr. Arthur Shoemaker) was used to considerable advantage as a glycolytic model

in hepatoma-free mice in connection with metabolic studies of the situation in the hepatoma 16650-RCN complex. In both RCN tumors there was a profound infiltration of the host livers accompanied by an overpowering glycolysis due to RCN cells.

6) A large number of primary hepatomas have been glycolytically studied under conditions comparable to those employed with the transplanted tumors. In most cases portions of adjacent apparently tumor-free host liver were simultaneously compared with the liver tumors. In some 100 primary liver tumors studied to date the glucolytic capacities of the tumors have been very significantly in excess of those occurring in the host livers or in livers of tumor-free mice. (Very limited apparent exceptions were found last year (see project NCI-334, 1970-71) in certain very large but non-necrotic primary hepatomas in 21 month old C_3HfB mice. An attempt to recreate these peculiar "Normalized" tumors is underway). However, the overwhelming amount of data obtained so far in these investigations support the view that the origin and progression of malignancy always involves an increased capacity to carry out glucolysis anaerobically, especially an increased capacity to utilize low levels of glucose and a decreased susceptibility to anti-insulin (steroid) regulation.

7) Studies of the ontogeny of hepatic glycolysis in normal livers from mice of varying ages (foetal 18-20 hrs to postnatal 20 hrs to 15 months) were extended and compared with primary and transplanted hepatomas with respect to glucolytic properties and anti-glucolytic hormonal regulation thereof. In hepatomas with respect to increasing affinities for glucose (lowered K_m) and decreasing susceptibility to hormonal regulation the tumor spectrum appears to represent the reversal of normal ontogeny i. e., a limited reversion to a neoembryonal state.

8) Comparative glycolytic profiles of different primary hepatomas from the same individual host animal were compared with each other and in some cases with other types of tumors in the same animal (e. g., mammary and lung tumors). Each type of primary tumor has shown its characteristic glycolytic profile independent of the other tumors in the same animal.

9) Glycolytic evaluations were continued in a number of transplanted mammary tumors (e. g., transplanted in BALB/c (30+ generations), BALB/c tp 1-3 generations, C57 BL and C57 BL (Moore) 1-8 tp generations; primary mammary tumors in C_3H, C_3HfB and $C_3HA^{vy}fB$ were also studied. Significant differences were found between various mammary tumors with respect to absolute rates of glucolysis, responses to insulin, steroids, etc.

Significance to Cancer Research: From studies in diverse types of normal tissues (kidney, brain, spleen, liver, etc.) and different types of tumors (melanomas, mammary carcinomas, tissue culture-originated sarcomas, lymphocytic and granulocytic leukemias, hepatomas, cholangioma, RCN's etc.) it is clear that essentially the same type of steroid-insulin control of

glucolysis can be demonstrated. Where the relevant non-malignant cells of origin have been available for comparison the derived cancer cells have shown not only increased rates of anaerobic glucolysis, but probably more important, decreases in susceptibility to steroid-induced inhibition of glucolysis. Similar progressive decreases in susceptibility to such glucolytic regulation were found in tumor spectra based on progressive anaplasia (e. g., S91 melanoma derivatives and Sanford tissue culture tumor clones). There is considerable evidence that glucolytic capacity plays a crucial role in the functioning of defensive cells of the RES. Successful chemotherapy may depend upon knowledgeable exploitation of differences in glucolytic regulation in cancer and in the normal defensive cells (RES).

Proposed Course of Research: In the recent literature insulin has been reported to exert its primary anabolic effects on cell metabolism via its role as an enzyme inducer. However, such effects on enzyme induction follow a lag period of at least several hours and may actually be secondary to the direct (minutes) effects on glucolysis at the level of the hexokinase reaction. Such stimulation of glucolysis may take place in growth-controlling regions of the cell (e. g., mitochondria, ribosomal regions of the cytoplasm). The occurrence of progressive resistance to steroid therapy as occurs in human prostate cancer and mammary cancer suggests the need for a better understanding of the possible anti-glucolytic role (or even pro-glucolytic role at very low concentrations) of steroids in metabolic regulation in such tumors. It is proposed that the lines of investigation presently underway in this project be continued.

Honors and Awards: None

Publications: None

Serial No. NCI-330
1. Laboratory of Biochemistry
2. Cytochemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Production of healthier cigarettes

Previous Serial Number: NCI-330 (plus 1965-66 NCI-331 and 332)

Principal Investigator: Dean Burk

Other Investigators: None

Cooperating Units: D. D. Tiggelbeck, Pittsburgh Activated Carbon Division, Pittsburgh; G. H. Cassels-Smith, J. S. Young Company, Baltimore, Md.; Vincent Lisanti, Council for Tobacco Research U. S. A., New York City; P. and R. Grimm, Delcron Co., New York City; Kurt Grob, University of Zurich, Switzerland.

Man Years:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: The development and application of cell metabolic techniques for identification and biological quantitation of harmful components in cigarette smoke, and the development of effective filters and air dilution devises for eliminating these components. This project most closely fits into the National Cancer Plan in Area 1 (To reduce the effectiveness of external agents in increasing the probabilities of development of cancer), Approach Element 5 (Application of current knowledge, and the development of immediately applicable information).

Methods Employed: Tumor and other cell materials are exposed in vitro to various types and quantities of smoke, and effects on cell metabolism, growth, or death determined, manometrically, microscopically, and by staining. Manometrically one measures effects on fermentation, respiration, and the Pasteur Effect, with tumor, normal mammalian cells, or yeast.

Major Findings: We have observed that large and readily measured quantities of gas are promptly eluted from various charcoals and charcoal filters upon simple addition of water or other liquids, and that the greater the amount of gas so produced from a given charcoal, the greater the effectiveness of the given charcoal in removing noxious components from cigarette smoke. A detailed study of such phenomena has been carried on in the hope of providing a new basis for selecting or making greatly improved charcoals for use in cigarette filters. It is well known that charcoals deterioriate markedly in effectiveness (up to two-thirds and more) in the course of days and weeks of cigarette shelf life, and our studies are aimed at ameliorating this situation where possible.

If 100 mg of our best available charcoal (activated and impregnated) is placed in the side-arm of a manometric vessel filled with air, with 3 cc of water in the main compartment of the vessel, and all brought to equilibrium in a constant temperature water bath, and then the charcoal and water mixed, up to some 500 mm^3 gas (NTP) are evolved within seconds. This unsuspectedly large quantity of gas is many times greater than the charcoal pore surfaces where its volume as condensate would be some 2000 times smaller. The evolved gas, under the defined conditions, is mainly air (N_2, 0_2), which, by adjunctive measurements, was found to contain no H_2, CO_2, CO, etc., although if acidified water instead of neutral water is employed for elution, then large amounts of CO_2 may also be evolved, even greater than the N_2 + O_2, and most notably so with charcoals that are alkaline. A secondary, irreversible phenomenon follows the elution of air, namely, a slow uptake of O_2, requiring hours or even days for completion, but amounting quantitatively to considerably less than the original air evolution. If the original vessel equilibration had been carried out not with air (or O_2), but with O_2-free N_2, H_2, He, or Ar, there was no reverse uptake of any of the gas immediately evolved upon mixing the charcoal and neutral water.

When either N_2, O_2, or Ar gas was used for the initial equilibration, approximately the same large quantities of gas were immediately evolved as when air was used, but now the gases given off were respectively N_2, O_2, or Ar. In other words, two or three minutes equilibration at most with such gases was enough to drive off the air held adsorbed within the charcoal (just as water could do so within seconds), and replace it with the particular equilibrating gas. Furthermore, upon equilibrating with any one of these three gases, and then further equilibrating with either of the other two yielded upon adding water, the gas last used for equilibrating; i.e., the exchange of gases onto the charcoal was not only rapid but also highly reversible. A much slower exchange occurs between water vapor and charcoal-bound air, over hours and days, without ever adding liquid water to charcoal, and this is what happens in a cigarette.

Now, if H_2 were used for the vessel equilibration, and water then added, only about one-sixth as much gas was eluted (being H_2), and with He only about one-twentieth. The basis of this decreased elution (compared to N_2, O_2, Ar) is not yet fully worked out, but presents interesting possibilities. As above, if upon first equilibrating with either H_2 or He, but finally re-equilibrating with N_2 (O_2, Ar), then the high N_2 (O_2, Ar) value for gas evolution was observed, again indicating rapid reversibility even with respect to gases producing low gas evolution compared to high evolution.

The above phenomena have been observed qualitatively with a great variety of charcoals, but with quantitative differentiation. Thus, at the other extreme from the activated, impregnated charcoal, a nonactivated, nonimpregnated charcoal produced only about one-eighth as much gas, and at the same time it was correspondingly far less active in removing noxious components from cigarette smoke, when tested by our fundamental, rapid, biological test system with e. g. Ehrlich ascites cancer cells on yeast cells (Proc. A. A. C. R., 10, 11, 1969; 11, 13, 1970; 13, 140, 1972). So far as tested, there has been an excellent correlation, among a variety of charcoals and charcoal filters, between ability to prevent metabolic harm from cigarette smoke, and ability to yield gas upon adding neutral water, the former test being biological and the latter physico-chemical.

Improvement of charcoals and charcoal filters by heating ("roasting") or desiccants. It is well known that charcoals placed as filters in cigarettes show marked deterioration in adsorptive capacity over days, weeks, and months of "shelf life," even when well sealed with cellophane wrapping. We have found that placing either charcoal or cigarettes containing charcoal filters for some 10 minutes at 240°F (or longer times at lower temperatures) greatly restores the abilities of such treated charcoals to yield greater quantities of gas upon adding water (as above) and likewise capacities to prevent biological harm from smoke when such charcoals* is in general greater, percentagewise, with the initially poorer charcoals, as might be expected. On the other hand, not all charcoals can be brought fully to the identically same high level of capacity of our best charcoals; in short a differentiation between various charcoals cannot be entirely eliminated by "roasting", even though very considerably reduced, and the beneficial effects of activation and impregnation remain qualitatively if not quantitatively the same, and this is likewise true with respect to "specific" adsorptions. Thus, at the lowest extreme, our nonactivated, nonimpregnated charcoal could only be brought to a gas evolvement of 200 mm^3/100 mg charcoal by the roasting process, compared to the some 500 mm^3/100 mg charcoal for our best activated, impregnated charcoal. As an intermediate instance, roasting Lark cigarette charcoal (either before or after its removal from commercial Lark cigarettes) raised its value from ca. 120 to 350 mm^3 gas evolved/100 mg charcoal.

*are placed in standardized cigarettes and then smoked. Such "recovery" in capacity

When charcoal was admixed with large-chunk silica gel desiccant in a small stoppered bottle, considerable improvement in capacity was noted in twenty four hours, and more after a week - obviously a much slower, but nevertheless in principle, effective method of "recovery." Whether a suitable desiccant (or buffer humectant) placed in a charcoal filter material in a cigarette would act advantageously or otherwise remains to be ascertained. Similarly, work is continuing to measure the rates of deterioriation of roasted charcoals put back into standardized cigarettes, to see what effective gain, if any, there might be. Presumably, the better the charcoal to begin with, the longer would any degree of protection persist. In any event, the present study shows clearly that a major component distilling from the tobacco to the charcoal filter in a cigarette is simply and largely water that then decreases the charcoal capacity for noxious smoke adsorption needed when the cigarette is actually smoked.

Significance to Cancer Research: In harmony with the frequently expressed view of the PHS Surgion General that cigarette smoking is the leading public health hazard about which something can be done, and that the most effective method of reducing this hazard re lung cancer, emphysema, heart ailments, etc., is to develop less hazardous cigarettes, two quite different modes of accomplishing this reduction have been studied by us by our new, simple, and fundamental methodology, one mode eventually involving largely passive public participation, and the other mode requiring active public participation. First, the most effective and available industrial charcoals and charcoal filters for decreasing cellular harm by cigarette smoke have been ascertained involving minimum particle size consistent with satisfactory cigarette draw (pressure drop), and large particle pore size (Proc. A. A. C. R., 10,11, 1969; 11, 13, 1970; Annual and other Reports, 1969-70). Second, commercially available cigarette holders that dilute smoke with adjustable proportions of air have been demonstrated to proportionally reduce cellular harm as the smoke/air ratio is decreased, in a manner subject to mechanical control and psychological adaptation on the part of, and at the will of, the smoker (Proc. A. S. B. C., 1971, Annual Report 1971). Industrial and institutional support of either or both of these modes could lead to notable reduction of cigarette smoke harm, on a large public health scale, and now a third method of experimental approach has been developed, as indicated in the foregoing paragraphs (cf. Proc. A. A. C. R., 13, 140, 1972). None of these three modes of approach were considered by Dr. G. B. Gori, Associate Director for Program, Etiology, NCI, in his summary talks on less hazardous cigarettes at the 2nd World Conference on Smoking and Health, London, Sept. 20-24, 1971, or at the Tobacco Research Program session on Carcinoma of the Lung held at Masur Auditorium, N. I. H., March 28, 1972.

Publications: None in common conventional form.

PHS-NIH
Summary Report
July 1, 1971 through June 30, 1972
Laboratory of Biology
NCI

Twenty-five papers have been published by the staff of the Laboratory of Biology during the year. Some of the findings reported in these papers and others not yet published are summarized here.

The team of Doctors Gantt, Stromberg, and Smith have continued their studies of tumor viruses with emphasis this year on methylases. This is relative to the hypothesis that an aberrant methylation of nucleic acids is a primary chemical event in some malignant transformations. Last year they found a specific methylase in avian myeloblastosis virus. Further studies of this and other viruses have now shown that the leukemia viruses have only N^2 guanine RNA methyltransferase activity while the sarcoma viruses have both N^2 guanine and 6-methyl-amino purine RNA methyltransferase activity. With the method developed by Stromberg for separating the virion from the capsule, it has been shown that the activity is not in the core of the virion, suggesting that a nucleic acid component in the host cell is the natural methyl group acceptor rather than the nucleic acid associated with the virus. Earlier observations by Gantt of a difference in methylase between neoplastic and normal cell lines together with the present observations suggest that both the viral induced and the spontaneous conversion may be the result of a common aberrant methylation event.

Studies of Smith and Gantt on mammary tumors indicate a higher methylase activity in mammary tumor virus (MTV)-induced mammary tumors, an intermediate activity in nodule inducing virus (NIV)-induced mammary tumors, and a low activity in those in "virus-free" BALB/c mice. This parallels the rate at which these tumors develop.

In their studies of A particles, Smith and coworkers have shown that there is apparently no relationship between intracisternal A particles and intracytoplasmic A particles. They do not share common antigens or proteins. The intracytoplasmic A particle appears to be related to the MTV B particle. Although electrophoretic patterns indicate that the two are distinct, there is cross reaction in complement fixation tests.

Work is advancing in the general area of the relationship of the RNA tumor viruses and the host genome (Heston, Vlahakis, Hoffman, and Smith). In hybridization studies with strain C3H-A^{vy}fB that has a high mammary tumor incidence from NIV although MTV is absent, it is shown that the A^{vy} gene is contributing to this high incidence but it is not yet known whether the gene has a direct influence on the virus.

To strengthen our ability in immunology Dr. Hoffman has been getting training in Dr. Old's laboratory at Sloan-Kettering particularly to learn the immunoflourescence test for MTV or NIV antigens. If we can test for the antigen in relatively young females we hope to get data on the provirus

theory in the transmission of MTV and if there is a provirus to map it in the host genome. This research on viral passage is one of the most important areas of research in viral oncogenesis today and we are fortunate to have the link with Old's laboratory.

Hoffman also has continued his basic biochemical genetic studies of mouse urinary proteins and catalase. Hoffman and Litwack have isolated four MUP-1 components but immunological cross reactivity between these isolated proteins indicated a similarity of structure. Carbohydrate was found only in component-1. Linkage analyses of the low liver catalase gene (Ce), and the acatalasemic gene (Cs) indicate that these are not the same gene, and probably not even on the same chromosome. Variations between liver, kidney, and erythrocyte catalase are being worked out.

Heston, Vlahakis, and Smith have produced and studied mammary tumors in C57BL/M mice with hypophyseal isografts. This strain is reported not to have MTV but to be relatively susceptible to the virus. The induced tumors do not have MTV B particles. This is one more section of information on the relationship of the host-genome, the virus, and hormonal stimulation in mammary tumorigenesis. The virus, although usually a factor, does not have to be present for mammary tumorigenesis.

In a study designed to give evidence as to whether the endogeneous murine leukemia virus (MuLV) is a direct cause of neoplastic transformation of mouse cells in vitro, Dr. Sanford and coworkers could find no correlation among 48 cell lines studied between presence of antigen or infectious virus and neoplastic transformation. It appears that MuLV is rather widely distributed in embryo cells of the C3Hf mouse but is probably not a single direct cause of spontaneous neoplastic transformation in vitro.

From the original observation that cells in media containing horse serum transform more readily than those in media containing calf serum, Evans and coworkers in the Tissue Culture Section continue to analyze this difference. Whether the horse serum comes from stallion, gelding, or mare makes a difference in rate of transformation, and the size of the molecule fraction also makes a difference. Further studies are aimed at ascertaining a hormone-like action in the difference in serums from different sexes.

The staff of the Tissue Culture Section continues basic work in cell nutrition. Some 15 new formulations of chemically defined media without serum or protein have been tested. Results indicate that specific media may be required for different specialized cells. Dr. Taylor particularly is studying the effect of peptones or peptone dialysates as serum substitutes for cell growth and analyzing their potential effect on malignant transformation.

Paralleling these nutrition studies are their continuous advancements in tissue culture instrumentation. A homeostatic culture system is under development which allows for monitoring and control of pO_2, pCO_2, pH, and glucose utilization, as well as allowing for automated fluid renewals or recirculation. The system has the potential of growing masses of cells in fluid suspension or as surface substrate cultures. Further development of Laminar Hoods is also underway. The hoods presently used have certain defects and

the approved Biohazards Hoods are not satisfactory for tissue culture work. A hood must, therefore, be developed that will not only meet biohazard standards but will also meet tissue culture technique standards.

In Heston's project on testing the anti-fertility drug "Enovid" for possible carcinogenic effects, although final analysis of the data has not been made, the following general statements can be given. 1) The epithelial changes in the cervix of BALB/c females attributed to Enovid in other preliminary studies, also occur in control females, and while they show some characteristics of malignancy they do not develop into frank tumors. The diagnosis is always microscopic. 2) Although some other preliminary studies with few animals may have suggested a link between Enovid and breast cancer, in the hundreds of females of 5 different strains on Enovid in this study there has been no evidence that Enovid at the dose preventing pregnancy either induces mammary tumors or causes them to appear earlier. 3) While ovarian tumors occurred particularly in C3HfB females, final tabulation will probably show no greater occurrence among the females taking Enovid than among the controls. 4) Small cortical adenomas occur in the adrenals of the BALB/c females but in no greater frequency in the Enovid groups than in the controls. 5) The only tumors increased by Enovid were the chromophobe adenomas of the pituitaries of strain C57BL. This confirms on a much larger scale Poel's observations in strain C57L. These results point out the advisability of large scale examination of pituitary glands taken at autopsy from women who had been on the "pill". Such pituitaries might be sent to a central laboratory like AFIP for examination and tabulation.

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2. Gantt, R. R., Stromberg, K. J., and Montes de Oca, F.: Specific RNA methylase associated with avian myeloblastosis virus. Nature 234: 35-37, 1971

3. Gantt, R. R., Smith, G. H., and Julian, B.: Base-specific methylase activity in RNA tumor viruses. I. Avian leukosis virion-associated RNA methylase(s). Virology, 1972

4. Gantt, R. R., Stromberg, K., and Julian, B.: Absence of RNA methylase in the AMV core. J Virol (in press)

5. Gantt, R. R., Montes de Oca, F., and Evans, V. J.: Methylation of satellite DNA in mouse neoplastic and non-neoplastic cell cultures. Submitted to Biochim Biophys Acta

6. Gantt, R. R., Stromberg, K., and Julian, B.: Localization of a specific RNA methylase in avian myeloblastosis virus. Submitted to J Virol

7. Hanley, C. and Hoffman, H.: Investigations of non-human primate hemoglobin: fetal hemoglobin. Med Prim 1970, 2nd Conf of Surgery & Med in Non-Human Primates (Karger, Basel, 1971), pp 239-255

8. Heston, W. E.: Genetic Factors in Tumorigenesis. In: RNA Viruses and Host Genome in Oncogenesis (P. Emmelot and P. Bentvelzen, eds.), North Holland Publishing Co., Amsterdam, 1972

9. Heston, W. E., Vlahakis, G., and Smith, G. H.: Mammary gland tumors in substrain C57BL/M and C57BL/He mice. J Nat Cancer Inst (in press)

10. Gantt, R. R.: In vitro tRNA methylation of paired neoplastic and non-neoplastic cell cultures. Cancer Res 31: 609-612, 1971

11. Hoffman, H. and Rechcigl, M., Jr.: Erythrocyte catalase in inbred mice. Enzyme 12: 219-255, 1971

12. Litwack, M. and Hoffman, H.: Murine major urinary protein: separation of subunits and determination of carbohydrate content. Submitted to Biophys Biochim Acta

13. Parshad, R. and Sanford, K. K.: Oxygen supply and stability of chromosomes in mouse embryo cells in vitro. J Nat Cancer Inst 47: 1033-1035, 1971

14. Reuber, M. D., Stromberg, K., and Glover, L. E.: Influence of age and sex in hepatocarcinogenesis induced by chemical carcinogens: ingestion of 3'-methyl-4-dimethylaminoazobenzene by Buffalo strain rats. J Nat Cancer Inst 48: 675-683, 1972

15. Rotherham, J., Price, F. M., Otani, T. T., and Evans, V. J.: Some aspects of the role of vitamin B12 and folic acid in DNA-thymidine synthesis in a neoplastic C3H mouse cell strain. J Nat Cancer Inst 47: 277-287,1971

16. Ritzky, L. P., Taylor, W. G., Pumper, R. W.: In vitro stimulation of rat liver cells by homologous partial hepatectomy serum. In Vitro 7: 1-7, 1971

17. Sanford, K. K., Jackson, J. L., Parshad, R., and Gantt, R. R.: Some evidence for an inhibiting influence of fetal bovine serum on "spontaneous" neoplastic transformation in vitro. J Nat Cancer Inst (in press)

18. Sanford, K. K.: "Spontaneous" neoplastic transformation in vitro. Acta Unio Internat Contra Cancrum. (in press)

19. Smith, G. H. and Wivel, N. A.: Isolation and partial characterization of intracytoplasmic A particles. Virology, April, 1972

20. Stromberg, K.: Surface-active agents for isolation of the core component of avian myeloblastosis virus. J Virol 9, 1972

21. Stromberg, K., Gantt, R. R., Wilson, S. H., Litwack, M., and Julian, B.: Localization of constituents in avian myeloblastosis virus. Proc Am Assoc Cancer Res, March, 1972

22. Stromberg, K., Litwack, M., and Desmukes, B.: Rapid purification and quantitation of avian myeloblastosis virus. Submitted to J Virol

23. Stromberg, K.: A new surfactant for isolation of the core of avian myeloblastosis virus (AMV). Fed Proc Fed Amer Soc Exp Biol 30: 110,1971

24. Taylor, W. G., Price, F. M., Dworkin, R. A., and Evans, V. J.: A simple device for planting replicate mammalian cell cultures. In Vitro 7:1972

25. Taylor, W. G., Taylor, M. L., Lewis, N. J., and Pumper, R. W.: Studies on a serum substitute for mammalian cells in culture. I. Biological efficacy of whole and fractionated peptone dialysate. Proc Soc Exp Biol Med 139: 96-99, 1972

Serial No. NCI-3208
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The study of the inheritance of mammalian proteins and their relationship to the incidence of tumors

Principal Investigator: Harold A. Hoffman

Other Investigators: Marcia Litwack, Wendel Neimann (New York Univ. Medical School), Gilbert H. Smith, and Warren Evans (LB,NCI)

Man Years:

Total:	5
Professional:	2
Other:	3

Project Description: Objective 3 Approach 3

Objectives: To study by methods appropriate to biochemical and quantitative genetics the inheritance of proteins in the mouse and other mammals and to determine the genetic and non-genetic relationships between the proteins and tumors. To determine the primary structure of the normal protein and the aberrant or mutant protein in order to define the gene or genes controlling their production and to determine the time of gene action during the process of neoplastic transformation.

Methods: In these investigations mice from 18 inbred strains and substrains, which represent at least 6 different independent lines of known genetic origin, are used as sources of proteins and tumors, and for the production of isologous antisera. New Zealand white rabbits of known immunoglobulin type are used for the production of heterologous antisera, and non-human primates of the order anthropoid are used as a source of protein.

Immunochemical methods, agar-gel-double diffusion and immunoelectrophoresis, are used in the detection and characterization of different proteins. Physical and chemical techniques of starch-gel electrophoresis, agar-gel electrophoresis, polyacrylamide-gel electrophoresis, and chromatography, both gel filtration and ion exchange, are used to isolate and classify different proteins. The fine structure and minute differences between closely related proteins are determined by peptide map analysis (fingerprints) and amino acid analysis.

Inheritance of murine urinary protein: The four components of the major urinary protein complex (Mup-1) have been isolated by a combined technique of gel-filtration and ion-exchange chromatography developed by Dr. Litwack. Antisera specific for component-2, produced by injecting purified component-2

protein into a rabbit from Mup-1a mice, and antisera specific for component-3, produced in a rabbit by injecting purified component-3 protein from Mup-1b mice, were compared with rabbit anti-whole Mup-1a and anti-whole Mup-1b by Ouchterlony analysis. The four antisera were reacted with whole Mup-1a, whole Mup-1b, and isolated components; complete immunological crossreactivity was observed between the isolated proteins regardless of the antisera used, indicating a similarity of structure between Mup-1 components. Carbohydrate was found only in component-1 by chemical analysis and by peptide map analysis.

Inheritance of tissue and erythrocyte catalase in inbred mice: Linkage analysis between the low liver catalase gene (*Ce*) and the acatalasemia gene (*Cs*) on linkage group V failed to establish linkage between these two genes. Using the lethal yellow (A^y) gene as a marker, the map distance between *Cs* and A^y by backcross analysis was 22.3 ± 3.9, while the *Ce* gene failed to give a significant chi square value for the 1:1 segregation with A^y. A linkage analysis between *Ce* and a second group V gene, pallid (*pa*), involving 147 F_2 animals failed to demonstrate a significant deviation from a 9:3:3:1 ration. These analyses establish that the liver *Ce* gene and the erythrocyte *Cs* gene are not one in the same and that they are probably not on the same chromosome.

Experiments to characterize the *Ce* gene, involving the inbred strains BALB/c,C57BL, and C57BL/6, and their hybrids, have produced some exciting results. First, the F_1 generation between the C57BL, high liver catalase, and the C57BL/6, low liver catalase, were low liver and the F_2 mice demonstrated a 3:1 low to high liver catalase distribution. The kidney and erythrocyte catalase, which is the same for these two strains, did not vary in the F_1 and F_2 generations. Secondly, first-generation backcross mice from the C57BL and C57BL/6 mating showed a 1:1 distribution for high and low liver catalase, but no segregation for the kidney and erythrocyte catalase. The cross between BALB/c, high liver, high kidney, and high erythrocyte catalase, and C57BL, high liver catalase, and intermediate kidney and erythrocyte catalase, produced an F_1 generation with intermediate liver, kidney, and erythrocyte catalase levels. The F_2 generation for this cross has not been completely analyzed; however, there are a significant number of F_2 progeny with extreme catalase activities which are outside the range of either inbred parent, indicating that there are new gene combinations in the F_2 which do not exist in either parent strain. The first-backcross generation progeny produced by mating the F_1 with the C57BL parent produced the most significant data to date. The kidney and erythrocyte catalase levels segregated into a 1:1 intermediate to high distribution indicating (1) a genetic correlation between the kidney and erythrocyte systems, and (2) that both the kidney and erythrocyte catalase levels are controlled by a single major locus. The liver catalase levels, on the other hand, segregated into 4 discrete groups, one of which is identical to the low liver catalase activities found in C57BL/6. It appears then, that the liver system is controlled by at least two loci which are genetically independent. If the two loci in question are the *Ce* and *Cs* loci, these backcross data are confirmed by the linkage data cited above. The second significant finding from these experiments is that these data confirm the earlier observation that the gene acting in the liver (probably *Ce*) is tissue specific whether other loci affecting catalase are segregating or not.

Collaborative studies with Dr. Warren Evans on the relationship between myloperoxidase activity from white blood cells and tissue and erythrocyte catalase activity are in progress. Our findings to date indicate that the myloperoxidase activity of white blood cells correlates with liver activity in the inbred strains of mice and that the acatalasemia gene Cs does not affect myloperoxidase activity of white blood cells. These results indicate that the Cs gene operates after the white blood cell and red blood cell lines have differentiated.

Proposed Course of Project:

Genetic characterization of the enzyme catalase system will continue. Production of three congenic strains, BALB/c, C57BL, and C57BL/6, for the Cs gene are in their second generation of backcrossing. These congenic lines are being produced in order to study the effect of the Cs gene on inbred backgrounds and to investigate the interaction between the Ce and Cs loci.

A study investigating catalase metabolism in normal liver tissue and in primary liver hepatoma has been initiated. A 40-60% decrease in catalase activity has been observed in primary hepatomas from mice of the strain C3H-A^{vy}/He. The present study is designed to estimate the rate of synthesis and the rate of degradation of the enzyme catalase in normal liver and in hepatomas.

Collaborative research with Dr. W. E. Heston on the genetic control of the transmission of MTV in inbred mice has been initiated. The study is designed to locate within the mouse genome the genetic site for the susceptability or resistance to MTV infection.

Publications:

Hoffman, H. and Rechcigl, M. Jr.: Erythrocyte catalase in inbred mice. Enzyme 12: 219-225, 1971

Hanley, C. and Hoffman, H.: Investigations of non-human primate hemoglobin: fetal hemoglobin. Med Prim 1970, 2nd Conf of Surgery & Med in Non-Human Primates (Karger, Basel, 1971), pp 239-255.

Litwack, M. and Hoffman, H.: Murine major urinary protein: Separation of subunits and determination of carbohydrate content. (Submitted to Biophys Biochim Acta)

Serial No. NCI-3251
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Testing for possible carcinogenic action of Enovid in mice.

Principal Investigator: W. E. Heston

Other Investigators: G. Vlahakis

Project Description: Objective 1 Approach 1

Objectives: The aim of this study is to ascertain whether or not the anti-fertility drug, Enovid, so widely used in women, has any carcinogenic effect when fed to mice.

Methods: The Enovid is fed in the NCI mouse food in three dosages:

5 µg Enovid/gm food
10 µg Enovid/gm food
20 µg Enovid/gm food

Assuming that a mouse eats 2 gm food/day, the daily doses would be 10, 20, and 40 µg Enovid/day. The lowest dose is approximately comparable on a weight basis to the dose given women.

In addition to the original four strains, A, BALB/c, C3HfB, and C57BL, used in this study we have also included strain C3H primarily to test the possible effect of Enovid on occurrence of mammary tumors. Only the highest dose of Enovid is given to the C3H females.

Results: Without final tabulation and analysis it appears that the only tumor influenced by the Enovid in the dosage used here to prevent breeding is the pituitary tumor. The occurrence of chromophobe adenomas of the pituitary is increased in C57BL/He females receiving "Enovid" over that found in the controls. This confirms on a larger scale observations of Poel made on strain C57L females. It appears that we are not going to be able to confirm an effect on cervical cancer or mammary tumors in mice due to Enovid. While some adrenal tumors have been observed in BALB/c mice, the incidence does not appear to be any higher in the "Enovid" females than the controls.

Significance of the Program to the Institute: Because of the importance of oral contraceptives in attempting to control the population explosion throughout the world, it is of utmost importance that someone at NCI investigate their possible carcinogenic effects in order that more educated decisions can be made regarding their use.

Proposed Course of Experiment:

During the coming year this study will be concluded.

Serial No. NCI-3253
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The influence of age and sex on hepatocarcinogenesis induced by chemical carcinogens

Principal Investigators: Kurt Stromberg and Melvin D. Reuber

Man Years:

Total:	2
Professional:	3/4
Other:	1-1/4

Project Description:

Reports have been made of studies on the influence of age and sex in hepatocarcinogenesis in Buffalo strain rats ingesting diethyldinitrosamine (Reuber, M.D. and Lee, C.W., J Nat Cancer Inst 41, 1133-1140, 1968) and N(4-fluoro-4-biphenyl)acetamide (Stromberg, Kurt, and Reuber, M.D., J Nat Cancer Inst, May 1970). A manuscript is in press on the age and sex dependency of hepatic tumors induced by 3'-methyl-4-dimethylaminoazobenzene, and a manuscript is in preparation with dimethylnitrosamine.

National Cancer Plan: Objective 3, Approach 3.

Methods: Inbred Buffalo strain male and female rats (12 to 16 rats per group) of ages 4, 8, 12, 24, and 52 weeks have ingested 0.0014% dimethylnitrosamine, and three months later the animals were killed and a complete autopsy was performed.

Results: Gross autopsy and light microscopic findings on rats ingesting 0.0014% dimethylnitrosamine inducated this carcinogen, when administered at this concentration, possessed minimal hepatocarcinogenic potential in any of the age or sex groups. However, male animals in younger age groups infrequently had sarcomas of the stomach and intestinal tract.

Significance of the Program to the Institute:

This series of pathologic studies is the first to evaluate the fundamental biologic parameters of age and sex in a uniform and comprehensive manner with representatives of three widely different classes of chemical carcinogens.

Publications: Stromberg, Kurt, and Reuber, Melvin D.: Influence of age and sex in hepatocarcinogenesis induced by chemical carcinogens: Ingestion of N-4-(4"-fluorobiphenyl)acetamide by Buffalo strain rats. J Nat Cancer Inst, May, 1970.

Reuber, Melvin D., Stromberg, Kurt, and Glover, L. E.: Influence of age and sex in hepatocarcinogenesis induced by chemical carcinogens: Ingestion of 3'-methyl-4-dimethylaminoazobenzene by Buffalo strain rats. J Nat Cancer Inst. 48: 675-683, 1972

Reuber, Melvin D. and Stromberg, Kurt: Influence of age and sex on hepatic lesions induced by chemical carcinogens: Ingestion of dimethylnitrosamine by Buffalo strain rats. In preparation.

Serial No. NCI-3254
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: An ultrastructural and biochemical characterization of avian myeloblastosis virus (AMV). I. Isolation of the core component. II. Identification and localization of enzymatic capacities in the virion. III. RNA and polypeptide composition of the core component. IV. Ultrastructural characterization of virion and core.

Principal Investigator: Kurt J. Stromberg

Man Years:
Total: 4
Professional: 1
Other: 3

Project Description:

The recent identification of a new surfactant for isolation of the core of avian myeloblastosis virus and the development of optimal methods for its use has opened the way to characterization of the two major subcomponents of AMV, a representative type C particle among the RNA tumor viruses. By surfactant-mediated removal of the envelope constituent of AMV, an assessment of the core constituent in ultrastructural and biochemical terms is now possible.

National Cancer Plan: Objective 1, Approach 4

Methods and Results: The methods which led to the selection of an appropriate surfactant for core isolation are described in a previous annual report (NCI-3247). Nonionic surfactant treatment with Sterox SL of AMV purified by rate-zonal centrifugation in Ficoll gradients permits isolation of a core component with a buoyant density of 1.27 g/cc in sucrose gradients. Use of a phosphate buffer instead of a Tris-HCl buffer during treatment enhances the yield of core protein. Core yield is linear over a range of starting intact AMV between 0.75 mgm/ml and 2.25 mgms/ml when the final concentration of Sterox SL is 1.5% (v/v). A five-fold increase in specific activity of endogenous RNA-dependent DNA polymerase is found in core preparations, while no activity of a recently described specific RNA methylase activity (Gantt, et al., Nature 234: 35-37, 1971) is found in cores. Polyacrylamide gel electrophoresis indicates that major structural proteins of the cores include gs1 and gs4, in the nomenclature of Fleissner (J. Virol. 8: 778-785, 1971). Transfer RNA activity is detected in core preparations. Studies are in progress to determine what proportion of the total 4S transfer RNA of intact AMV is packaged in this core component. This has importance insofar as it brings knowledge to bear on the role of the 4S transfer RNA in the virus life cycle. Is

there fortuitous incorporation of cellular 4S tRNA into the virus during virus assembly at the cell membrane, or in contrast, does the core location of this constituent imply that it is virus-coded and consequently essential for completion of the viral-life cycle? In collaboration with Doctors Ron Luftig and Vincent Marchesi, a high resolution electron micrographic examination of the virion is planned which will use the techniques of negative staining, freeze-etching, and the Kleinschmidt procedure.

Significance of this Project to the Institute:

A rapid, simple, and quantitative method has been developed for separation of the principal components of a representative C-type RNA tumor virus. This procedure will assist characterization of what is "host-contributed" (envelope?) and what is "virus-specified" (core?) in the AMV virion. Utilizing this methodology a rigorous examination and localization of virion and core constituents is currently in progress.

Publications:

Stromberg, K.: A new surfactant for isolation of the core of avian myeloblastosis virus (AMV). Fed Proc Fed Amer Soc Exp Biol 30: 1100, 1971

Gantt, R. R., Stromberg, K., and Montes de Oca, F.: A specific RNA methylase associated with avian myeloblastisis virus. Nature 234: 35-37, 1971

Stromberg, K.: Surface-active agents for isolation of the core component of avian myeloblastosis virus. J Virology 9: 1972.

Stromberg, K., Gantt, R. R., Wilson, S. H., Litwack, M., and Julian, B.: Localization of constituents in avian myeloblastosis virus. Proc Am Assoc Cancer Res, March, 1972

Stromberg, K., Litwack, M., and Desmukes, B.: Rapid purification and quantitation of avian myeloblastosis virus. Submitted to J Virology

Gantt, R. R., Stromberg, K., and Julian, B.: Localization of a specific RNA methylase in avian myeloblastosis virus. Submitted to J Virology

Stromberg, K., Gantt, R. R., and Wilson, S. H.: Enhancing conditions for isolation of the core of avian myeloblastosis virus. In preparation

Stromberg, K. and Litwack, M.: Localization of the 4S transfer RNA of avian myeloblastosis virus. In preparation

Stromberg, K. and Hurley, N.: Organization of the polypeptides in the virion and core component of avian myeloblastosis virus. In prep.

Stromberg, K., Luftig, R., and Marchesi, V.: An electron microscopic study of avian myeloblastosis virus. In preparation.

Serial No. NCI-3255
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: A correlative light and electron-micrographic study of hepatocarcinogenesis induced by chemicals

Principal Investigators: Kurt Stromberg, Melvin D. Reuber, and Gary Williams

Man Years:
Total: 2
Professional: 1/2
Other: 1-1/2

Project Description:

Electron microscopic studies have failed, perhaps due to lack of a model system, to reveal pathognomonic ultrastructural features which distinguish normal or hyperplastic cells from chemically-induced neoplastic cells. Two projects, one in vivo and one in vitro, were developed as model systems to look for early and specific morphologic changes in neoplasia. It is known that hyperplastic nodules induced in rats by N-2-fluoroenyldiacetamide progress to small hepatic carcinomas. The objective of this in vivo project is to evaluate this progression, which has been studied at the light micrographic level (Reuber, M.D., J Nat Cancer Inst 34, 697-723, 1965), in terms of a correlative light and electron microscopic study that uses tumor transplantation for biologic confirmation of the morphologic diagnosis. The in vitro project is based on the development by Dr. Gary Williams of a tissue culture cell line which retains characteristics of hepatocytes. This permits a morphologic evaluation of a limited number of cells at periodic intervals after exposure to chemical carcinogens and before subsequent placement in recipient rats to determine the malignant potential of the cells.

National Cancer Plan: Objective 3, Approach 1

Methods: In the in vivo study three-month-old male AxC inbred rats were fed 0.025% N-2-fluoroenyldiacetamide in a semi-synthetic diet for 16 weeks. Between the end of the 20th week and the 50th week autopsies were performed on several rats at successive 3-week intervals. Those hepatic lesions which were 1 centimeter or less in diameter, and grossly appeared to be either hyperplastic nodules or small carcinomas were bisected. One-half was divided and transplanted into two recipient rats; a tiny piece of the other half was prepared for electron microscopy, while the remainder was prepared for light microscopy. The use of thick plastic sections, about one micron in thickness and stained with toluidine blue, permitted precise correlation between these two conventional forms of morphologic evaluation and overcame the problem of random selection of cells.

The methods of in vitro growth of epithelial cells of hepatocytic origin by Dr. Williams is described in Experimental Cell Res 69: 106-112, 1971.

Results: Preliminary findings in the rat hepatoma system include 1) irregular swelling of lysosomes; 2) an increase in thickness of the plasma membranes which frequently balloon out as protrusions into the bile canaliculi; and 3) the occurrence of bizarre cytoplasmic structures, probably lipid accumulations, which are at times enveloped by concentric layers of smooth membranes near the region of the Golgi apparatus. Whether these findings represent toxic effects of the carcinogen and are associated with regression, or are a characteristic phase of malignant change will be determined by the transplantation results and more extensive morphologic evaluation.

Electron microscopic observation of the tissue culture cell line of Dr. Williams reveals the following salient ultrastructural characteristics: 1) a homogeneous population of epithelial cells in which the endoplasmic reticulum is saculated; 2) a frequent observation of cytoplasmic fibrils interdigitating with communicating cells; and 3) microbodies with crystal formation in nucleoids.

An outstanding finding was a correlation between morphology and histochemical evidence of various enzymatic activities in relation to the growth state of the cell. When attaining a stage of confluence (about 10 days), the cell lines contained microfilaments, few microbodies, and little evidence of enzymatic activity. When maintained beyond confluency (about 15 days), there was a dramatic increase in the frequency and definition of microbodies, as well as acquisition of over 10 enzymatic capacities characteristically associated with hepatocytes. Thus, it appears that the degree of confluency (or level of contact inhibition) induces the cell line to differentiate from a proliferating state to a functional state. In view of the enzymatic characteristics and morphologic appearance of hepatocytes and the persistence over long-term culture condition of a diploid chromosome number of 42, these cell lines are among the first to represent in vitro growth of epithelial cells of hepatocitic origin.

Significance of this Project to the Institute:

Technicial procedures and model systems were developed to detect the existence of morphologic correlates to carcinogenesis induced by chemicals.

Publications:

Williams, Gary M., Kroes, R., and Stromberg, K.: Properties of epithelial-like cells from rat liver in long-term cell cultures. In preparation.

Serial No. NCI-3256
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project title: Ultrastructure and biology of mammary tumor virus - host cell interactions

Principal Investigator: Gilbert H. Smith

Other Investigators: W. E. Heston, R. Raymond Gantt, Nelson A. Wivel, and Marcia D. Litwack

Collaborating Units: Harvey Ozer, LB, NCI
George Vlahakis, LBGY, NCI

Man Years:
Total: 5
Professional: 3
Other: 2

Project Description: Objective 3, Approach 2

To elucidate the aspects of cell-virus interaction in mouse mammary tumor virus infection.

Major Findings:

With Dr. W. E. Heston, experiments are continuing to evaluate the influence of the A^{vy} gene on the development of mammary tumors in mice. The approach was to produce segregants of the A^{vy} gene in backcross offspring of C3H/StWi x C3H-A^{vy}fB hybrids. Earlier results predict that the A^{vy} segregants would develop spontaneous mammary tumors at twice the percentage as the agouti segregants if the effect of the A^{vy} gene is the primary factor in the development of a high incidence of spontaneous mammary cancer in MTV-free C3HA^{vy}fB females. Preliminary results indicate this, however the number of mice with tumors is still too small to reach a final conclusion.

In collaboration with Dr. Nelson A. Wivel a method for the isolation of intracytoplasmic A particles from spontaneous mammary tumors and from testicular interstitial cell tumors has been developed. A paper describing the isolation procedure and some characteristics of the isolated particles has been accepted for publication in Virology.

In view of the widely-held belief the intracytoplasmic A particles represent intracellular precursors (nucleocapsid core) of the mouse mammary tumor virion (MTV). Studies were initiated to compare the properties of these particles to B particles (MTV virions).

SDS-gel electrophoretic analysis of A particle isolates were carried out. Several major proteins have been separated. Comparison of the electrophoretic patterns of A particle proteins with those obtained with purified MTV have shown them to be unique, i.e., distinct from one another. In spite of this, complement fixation tests carried out with A particles or with anti-A particle antibodies and with B particles and anti-MTV antibody, in cooperation with Dr. H. Ozer, Laboratory of Biochemistry, show a distinct cross-reaction between A and B particles which is not readily explained by the presence of anti-specie activity in the antisera. Studies to resolve these questions are in progress.

In collaboration with Dr. Marcia Litwack, RNA size analysis of the cytoplasmic A particle preparations are under way. Comparison of these results with those obtained with MTV preparations will be made. Results are preliminary.

Bioassay experiments on purified intracytoplasmic A particles are in progress. Sixty newborn virus-free BALB/c/He mice were inoculated with freshly prepared isolates. The mice will be observed as breeders for the appearance of MTV-specific antigens in the milk and for the appearance of mammary tumors. Early results indicate a low to negligible titer.

Nucleic acid methylase activity in normal, neoplastic, and preneoplastic mouse mammary tissues and in some of the murine and avian RNA tumor viruses have continued in collaboration with Dr. R. Raymond Gantt. Early results indicate higher methylase activity in MTV-induced mammary tumors, an intermediate activity in NIV-induced mammary tumors, and a low activity in the mammary tumors (which arise at low frequency) in "virus-free" BALB/c strain mice. Attempts to translate these results to normal lactating tissue have been troublesome, therefore studies were directed at the virion to examine it for the presence of a specific methylase (sequel to the observation of Gantt, et al. that avian myeloblastosis virions contain N^2,methyl guanine transferase). These studies are still in progress. In addition, the preceding observations have been extended to two other murine oncogenic viruses and to Rous Sarcoma Virus. A manuscript describing that latter observation has been submitted for publication.

In collaboration with Dr. Nelson Wivel, immunologic and SDS-gel electrophoretic analysis and comparison of intracisternal A particles and intracytoplasmic A particles were performed with the cooperation of Dr. H. Ozer in the Laboratory of Biochemistry. The results strongly indicate that no common antigen or protein is shared between the two types of A particles. A manuscript describing these studies is in progress.

Publications:

Gilbert H. Smith and Nelson A. Wivel: Isolation and partial characterization of intracytoplasmic A particles. Virology, April, 1972

W. E. Heston, G. Vlahakis, and Gilbert H. Smith: Mammary gland tumors in substrains C57BL/M and C57BL/He mice. J Nat Cancer Inst (In press)

R. Raymond Gantt, Gilbert H. Smith, and Barbara Julian: Base-specific methylase activity in RNA tumor viruses. I. Avian leukosis virion-associated RNA methylase(s). Submitted to Virology.

Serial No. NCI-3259
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Host genome-viral relationship in mammary tumor development in mice.

Principal Investigators: G. Vlahakis and W. E. Heston

Other Investigators: Gilbert H. Smith and Gabrielle R. Neises

Man Years:
Total: 4-1/2
Professional: 1
Other: 3-1/2

Project Description: Objective 1, Approach 4

Work with mammary tumors in mice continues with special interest being centered upon the relationship between the genetic factors and the viral influence in the development of these tumors.

In previous observations it had been emphasized that the viable yellow gene (A^{vy}) which characterizes strain C3H-A^{vy}fB undoubtedly plays an important role in the development of mammary tumors in the strain. One of the possible actions of the A^{vy} gene may be to enhance the usually poor oncogenic ability of NIV (nodule inducing virus) which strain C3H-A^{vy}fB harbors. Another possibility may be that the A^{vy} gene is acting in some other way, for example, on the mammary gland itself, to account for the increased susceptibility to tumor development in this gland.

Strain C3H-A^{vy}fB which has a ninety percent spontaneous mammary tumor incidence, in the absence of the milk borne mammary tumor virus (MTV), was outcrossed to the agouti strain C3H/StWi, in which mammary tumors have ceased to develop, and in which no MTV or NIV has been demonstrated. The F_1's were then backcrossed to C3H/StWi to produce two classes of animals: viable yellow (A^{vy}A) and agouti (AA). Both groups will have the variant mammary tumor virus, NIV, that is passed vertically at the time of conception by the C3H-A^{vy}fB parent. The NIV of strain C3H-A^{vy}fB is transmitted by the male parent as readily as by the female parent.

Results:

With the experiment still in progress, thus far the results have been as follows. Two large spontaneous mammary tumors have occurred in the A^{vy}A females, one at 11 and one at 15 months of age. In addition, there are five other possible mammary tumors. Among the AA females only one very small

mammary tumor was found at necropsy at 14 months of age. There is in addition one other possible tumor. Many of the $A^{vy}A$ females, however, are dying from multiple hepatomas and there are now approximately half the number in this group as compared with the AA females. These results suggest to us that the $A^{vy}A$ backcross females are more susceptible than the AA backcross females, to mammary tumor development, thus providing further evidence for an increased genetic susceptibility among animals with the viable yellow gene.

The NIV in strain C3H-A^{vy}fB as compared with NIV in the comparable strain C3HfB, where the mammary tumor incidence is approximately forty percent, may represent a more potent NIV with a higher tumorigenic capacity. Therefore, we have been attemtping to develop a strain BALB/c (which has a low mammary tumor incidence and which does not have MTV or NIV) with NIV from strain C3H-A^{vy}fB, and a strain BALB/c with NIV from strain C3HfB. If successful, this will enable us to compare the NIV's from the two strains in genetically similar hosts, in relation to mammary tumorigenesis.

The lines of mammary tumor viruses that occur in the different mouse strains continue to be of special interest to us. We now have more information on strain BALB/cfA (with AMTV) to compare with MTV's from strains C3H and DD, in genetically similar hosts.

Results

The MTV from the high mammary tumor strain C3H is slightly more potent than either MTV's from strains A and DD. The MTV's of strains A and DD however, are apparently similar in potency. For example, the mammary tumor incidence in BALB/cfC3H (with C3H MTV) is approximately 100%, with tumors occurring at an average age of 7.1 months. In both BALB/cfA (with AMTV) and BALB/cfDD (with DD MTV), the mammary tumor incidences are approximately 97%, and the tumor ages for BALB/cfA and BALB/cDD are 9.4 and 9.3 months respectively.

Strain BALB/cfC3H females even when maintained as virgins have an approximate mammary tumor incidence of 88% with an average tumor age of 11.2 months.

We also will have information on mammary tumors arising in virgin BALB/cfA and BALB/cfDD females. Thus far, three mammary tumors have occurred in virgin BALB/cfA females, one at 10, one at 11, and one at 12 months of age.

Recently it has been reported that cell free homogenates of mammary glands from old uninfected BALB/c females, that have a low spontaneous mammary tumor incidence, when injected into young BALB/c females, resulted in the occurrence of a high percentage of mammary tumors at 6 months of age. We are planning to do this same experiment with our own strain BALB/c females.

It also seemed worthwhile to apply this test to some of the large adenocarcinomas of the mammary gland, that occasionally arise spontaneously in strain BALB/c and are readily transplantable. Most of the mammary tumors in BALB/c are quite small, observed only at necropsy, and are diagnosed as adenoacanthomas.

Extracts were made of two mammary tumor transplants in strain BALB/c and injected into young virgin females, and are now being observed for the occurrence of mammary tumors.

Substrain C57BL/He which is one of the most resistant mammary tumor strains, is genetically very resistant to the mammary tumor virus, losing it in a generation or two after its introduction. The genetically similar substrain C57BL/M of Dr. Dan H. Moore at the Institute for Medical Research, Camden, New Jersey, that is susceptible to the mammary tumor virus, has now been shown to be more susceptible than C57BL/He, to the development of mammary tumors. It is therefore of interest to determine how well MTV survives in C57BL/M. Accordingly, C57BL/MfC3H (with C3H MTV) has now been developed. We hope to have some information on this foster nurse strain during this year.

In collaboration with Dr. Melvin Reuber of the University of Maryland Medical School, we are planning to publish a paper on the spontaneous tumors of the uterus that arise in our female mice. Such a publication should prove useful to those investigators who are working with these tumors in mice and possibly other rodents.

Significance of Program to the Institute:

The A^{vy} gene of strain C3H-A^{vy}fB is an example of the role of genes in tumorigenesis. The spontaneous mammary tumor incidence of strain C3HfB is raised from approximately 40% to 90% in the comparable strain C3H-A^{vy}fB. Similarly, the occurrence of spontaneous hepatomas is markedly increased in strain C3H-A^{vy}fB over that of strain C3HfB in both males and females. Tumors result from the interaction of a suitable genotype with hormones and viral agents. By the manipulation of the different MTV's with varying genotypes of mouse strains as we have done, we can study the relative influence of each in tumorigenesis.

Publications:

Heson, W. E.: Genetic Factors in Tumorigenesis. In: RNA Viruses and Host Genome in Oncogenesis, (P.Emmelot and P.Bentvelzen,eds.), North Holland Publishing Co., Amsterdam, 1972.

Serial No. NCI-3260
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on mammary tumors in resistant strain C57BL mice

Principal Investigator: W. E. Heston

Other Investigators: G. Vlahakis, G. Smith

Man Years:
Total: 1/2
Professional: 1/4
Other: 1/4

Project Description: Objective 1 Approach 4

Objectives: The aim of this study has been to attempt to induce mammary tumors in Moore's line of strain C57BL mice (C57BL/M) with pituitary isografts, to compare the induction in this line with that in our strain C57BL/He and to see if the tumors of either line carry the mammary tumor virus.

Methods: Forty C57BL/M females were used for this study. At from 2 to 3 months of age each female received under the capsule of the kidney isografts of 5 pituitaries from C57BL/M females of comparable age. The females were then set aside as virgins to see whether or not they would develop mammary tumors. For comparison 15 C57BL/He females likewise each received isografts of 5 pituitaries from other C57BL/He females of comparable age. Tumors that have arisen in the two lines have been examined with the electron microscope by Dr. Gilbert Smith to see if they contain viral particles.

Results: This study has shown that strain C57BL/M mice that Dr. Dan Moore has shown to be able to propagate the mammary tumor virus better than strain C57BL/He can is contrary to Moore's observations also susceptible to the induction of mammary tumors with hypophyseal isografts, and more susceptible than C57BL/He. All of the induced tumors of both substrains were adenoacanthomas. A few spontaneous mammary tumors have occurred in breeding C57BL/M females - a slightly higher incidence than in C57BL/He. Some of the spontaneous tumors have been adenocarcinomas. There was no morphological evidence for the presence of "derepressed" MTV in any of the induced tumors, and no evidence of mammary tumor virus particles in the C57BL/He spontaneous tumors. Some extracellular particles and budding forms were observed in the spontaneous C57BL/M tumors. Some of these were C particles but we are not certain that any could be the B particles of MTV.

Significance of Program to the Institute: These findings are of significance as they fit into our general knowledge of the genetics of mammary cancer in mice. It did not seem possible that Moore's strain of C57BL would not develop

mammary tumors with hypophyseal isografts since he had found that females of the strain could readily propagate the virus. Our findings that they do develop mammary tumors with hypophyseal isografts now fits into our general concept of a correlation between the degree by which a strain can propagate the MTV, the degree of susceptibility to induction of mammary tumors with MTV, and the degree of susceptibility to induction of mammary tumors by other means, in this case hypophyseal isografts. Failure to find MTV particles is further evidence that MTV is not always necessary for induction of mammary tumors in mice.

Publications:

Heston, W. E., Vlahakis, G., and Smith, G. H.: Mammary gland tumors in substrain C57BL/M and C57BL/He mice. J Nat Cancer Inst (In press)

Proposed Course: This study has now been concluded and the results submitted for publication.

Serial No. NCI-3261
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Chemical and ultrastructure studies on cytoplasmic DNA

Principal Investigator: Gilbert H. Smith

Other Investigators: Gabrielle R. Neises, collaborative research with Dr. Walter C. Schneider and Dr. William R. Kidwell

Project Description: Objective 3 Approach 1

Chemical and autoradiographic studies on the localization of DNA to membraneous components of the cell cytoplasm are in progress. Superovulated rat ovaries from immature animals are collected at various times after stimulation with luteotrophic hormones. Cellular ultrastructure and morphologic differentiation are compared with the appearance and quality of known microsomal enzymes. In addition, autoradiographic procedures are used to localize incorporated H^3-labelled nucleotides during these developmental stages.

Serial No. NCI-3211 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies, by tissue culture and cell culture methods and by amplifying methods, of the malignant cell and of the normal cell from which it arises; studies on the mechanism of transformation of the normal cell into the malignant cell; studies on the characterization and identification of such cells; studies on associated significant subjects.

Principal Investigators: V. J. Evans, K. K. Sanford, R. R. Gantt, W. G. Taylor, F. M. Price, and M. L. Taylor (Guest Worker and Research Fellow)

Other Investigators: See individual project reports Nos. 3212, 3213, 3234A, 3257 and 3262.

Man Years: July 1, 1971 through June 30, 1972

Total	18
Professional	10
Other	8

Project Description: See Project Title above, (National Cancer Plan Objectives and Approaches for Tissue Culture Section, 2.4, 3.1, 3.2, 4.1, 4.2; and secondarily 5.5, 6.1, 6.2, 6.3, 6.6)

Outline of Activities:

The long-term objectives of the personnel of the Section are indicated in the general title above and are described in detail in the individual projects. To carry out the objectives considerable time has been spent in the past years and will continue to be spent as required in order to maintain and develop superior technology and instrumentation, to advance current problems, particularly those related to the National Cancer Plan's 7 objectives, and to anticipate maintaining eminence in the primary interests.

Some of the unusually time consuming details related to evaluating equipment and supplies for long-term large-scale antibiotic-free tissue culture work are mentioned because they have resulted in recently designed equipment and quality control supplies that others outside the laboratory have found useful: Such things as unique glassware washing equipment and all-glass water distillation units. Stainless steel autoclaves operated with a special steam generator and heat exchanges for distilled water steam have been tested and work effectively. Two precise electronically controlled constant temperature rooms have been designed, constructed, and tested; these have been

copied both within and outside the NIH. The primary source water distillation unit is supplying adequate volumes of distilled water for washing but line connections apparently were unsatisfactorily tinned and will require some short line replacements to correct high copper content.

Currently still under need of correction are the so-called laminar flow clean hoods used for renewal of nutrient fluids on cell cultures. These were purchased from lowest bidder several years ago prior to completion of Building 37. Subsequently, new more satisfactory types have been designed. The ones currently in use have been found to be defective in the following respects: They build up high heat intensity in areas to 110°F and the air conditioning engineers to date have found no way to dissipate this. The perforated design table in these boxes is such as to make it difficult to keep clean or to clean the boxes, and more important from the workers' point of view, the present hoods are not safe for handling biologically hazardous material. Also, recent testing indicated construction is so poor that 1) air leaks were found between gaskets on filters and filter frames, 2) air leaks occur around lights, 3) sliding sash is not gasketed between sash and frame and pulls room air into bench, 4) aerosols are thrown onto worker because air is positive with respect to room, 5) accessibility for maintenance of filters is poor because of type of expanded metal screen used to support filters. There are additional other defects which will follow in an environmental services report. These hoods may have to be replaced to correct ultimately the defects mentioned, particularly the one of great vulnerability on handling of biohazardous material. Considerable time has been devoted to exploring substitutes for these laminar flow tissue culture hoods that would be both biohazard hoods for investigators' safety as well as effective for the satisfactory culture of cells, tissues, and organs in vitro. Boxes currently available and satisfactory for biohazard work are still cumbersome for use in modern tissue culture work and many tend to trap heat, as noted above, build it up around cultures, and expel it into operator's face. Now the rigid standards currently used by NIH are blocking the opportunity for individual labs to test products and find ones safe and suitable for non-biohazard work. The present standards are based only on needs for virologists and seriously handicap cell biologists' needs and the opportunity for them to run surveys and on the site tests on newly developing equipment.

Sources are being sought and some tests are under way on gelding horse serum to supplant the horse serum from horses maintained on the NIH farm at great expense and less than optimal conditions judging from the horse serum recently obtained. Our commercial source looks most excellent and the quality control tests are under way.

The microcinematograph has been purchased and is on hand. The instrument will be used to analyze in more detail dynamic morphology and growth characteristics of living non-malignant and malignant cells under diverse culture conditions. The temperature control system for the cultures is not yet calibrated for maximum efficiency.

The homeostatic perfusion culture system alluded to last year is fairly well under way and details are to be found in Serial No. NCI-3262.

A compact lab bench type liquid nitrogen freezer for preservation of cell lines, steel lined for safety, and with a custom designed inventory system, has been designed and purchased. This unit will undoubtedly have wide use in the field of cell preservation because of its safety features and flexibility of use and compactness for crowded laboratories.

Serial No. NCI-3212, Project 1 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project 1, Title: Studies of cell nutrition and metabolism in vitro.

Principal Investigators: V. J. Evans and F. M. Price

Other Investigators: H. A. Kerr, R. A. Dworkin, R. R. Gantt, W. G. Taylor, K. K. Sanford.

Man Years: See Serial No. NCI-3211.

Cooperating Units: Nucleic Acid Section, Laboratory of Biochemistry, NCI

Project Description: National Cancer Plan 2.4, specifically 2.4.3.3 and 2.4.3.8

A program to determine the nutritional and physico-chemical needs of cells in vitro for better utilization of such cells in a variety of problems. Primarily the cells are for the study of the phenomenon of spontaneous malignant transformation in vitro. The program also contributes to advancing tissue culture technology and to supplying basic contributory knowledge for biologic research materials, specifically mammalian cells, including human cells.

Objectives:

1. To improve tissue culture technology so that more cell types including human cells can be cultured for long-term in protein-free or protein-supplemented media without experiencing the usual degenerative changes.

2. To improve media, cultivation techniques, and separation methodology for large-scale production of normal and malignant human and mammalian cells.

3. To improve culture conditions of cells so that rigidly controlled cells can be used to study the differences between nonneoplastic and malignant cells at the nutritional, metabolic, and morphologic levels.

Methods Employed:

Freshly explanted mouse cells, long-term mouse cells, freshly explanted human embryo cells, and long-term human diploid or quasi diploid cells were grown in media of varying formulations with or without protein supplement. Substances used as supplements were: human α 1 protein; horse and bovine serum albumin; soy bean protein extract; large and small molecule fractions of horse and fetal bovine serum; gelding, mare and stallion serum; fetal bovine serum; and Witte's peptone and a dialysate of Bacto-peptone.

Also, the effect of 1% oxygen on cell growth was tested for freshly explanted mouse and human embryo cells and on one commonly used human diploid cell line, Wi-38.

Major Findings:

1. Development of a chemically defined medium for growth of freshly explanted and long-term cell lines.

a. Growth of primary cultures: Attempts have been made to improve the formulation of our culture medium in order to provide a medium which will support growth of primary cultures without the use of serum or protein hydrolysates. At the same time we were trying to eliminate unnecessary ingredients. Major changes thus far have been: 1) to change the buffering capacity of the medium to prevent an alkaline pH by decreasing the amount of bicarbonate and adding disodium phosphate; 2) to increase the concentration of glucose, several amino acids (particularly L-glutamine) and the B vitamins and vitamin E; 3) the addition of sodium pyruvate and dimethyl sulfoxide; 4) to delete from the formulation 3 amino acids, 6 coenzymes, and 3 nucleic acid precursors.

Results thus far have been encouraging, although no established or long-term cell lines have been obtained. Our best improved medium to date has supported growth of primary C3H mouse embryo cultures for 9 culture generations over a period of approximately 11 weeks. This compares favorably with the chemically defined medium, NCTC 135, which we have been using which has supported growth of primary mouse embryo cultures for 2 or 3 culture generations before they finally died out or lived indefinitely, but only with assiduous efforts, usually employment of strong adaptation pressures predicated upon constant observation. It appears from our results so far that freshly explanted cells can grow in completely defined medium, but a high percent of the cells are more fragile upon subculturing so that there is an ultimate decrease of the cell population below minimum inoculum numbers.

b. Growth of established cell lines: A quantitative study was made to determine the effect of three NCTC media formulations on the growth of strain L cells. The media used were NCTC 135, 145, and 149. These media differed from one another mainly in amino acid and vitamin content and salt concentration. Flasks were inoculated at a population of 180,000 cells per flask. After 3 days of growth the population was about 500,000 cells/flask in all three media. After 5 days growth the population of cells in medium NCTC 135 was 800,000/flask, in medium NCTC 145 was 1,000,000/flask, and in medium NCTC 149 was 1,000,000/flask. After 7 days, however, the population in NCTC 135 was 1.9 million/flask, that in NCTC 145 1.5 million/flask, and in NCTC 149 1.7 million/flask. It appears from this that these cells initially (i.e. up to 5 days) grow slightly faster in the two newer formulations, NCTC 145 and 149. Since strain L cells have been grown continuously for many years in NCTC 135, it may be that some adaptation is required in order to have a greater overall rate of proliferation in a medium different from NCTC 135.

Several newer media formulations have now been prepared and used for some culture work, NCTC 161 and 162. These media differ from the others in that they contain a much larger amount of amino acids and vitamins. Strain L cells

have been maintained in medium NCTC 161 for 17 culture generations with superior growth. Comparative quantitative studies with this strain will be carried out to determine influence of adaptation.

2. Growth of freshly explanted cells in medium supplemented with human α1 protein, horse and bovine serum albumin, soy bean protein extract, peptone, or peptone dialysate.

Initially all studies were carried out with NCTC 135 as the basal medium. When used at a protein concentration equivalent to that of whole serum, horse or bovine serum albumin would not replace whole serum as a growth supplement to NCTC 135. Freshly explanted mouse cells in medium supplemented with bovine serum albumin grew for 3 culture generations before dying out. Cultures in horse serum albumin could not be subcultured even once. Cultures grown in medium NCTC 135 supplemented with human α1 globulin also did not live for more than 3 culture generations. Cells grown in medium with a combined supplement of bovine serum albumin and human α1 protein lived through 4 culture generations before their growth rate declined rapidly. The life of the cultures, however, could be prolonged by substituting NCTC 161 (described in objective 1 above) for NCTC 135. To date 2 cell lines which were almost completely depleted of living cells in the 4th culture generation in NCTC 135 supplemented with 1 protein and bovine albumin have recovered enough to go through 2 more subcultures and are presently growing in the 7th culture generation.

Medium NCTC 135 was also supplemented with 5 concentrations of soy bean protein extract to determine whether this protein supplement would replace serum. Growth of freshly explanted mouse cells was very poor in the supplemented medium regardless of concentration and all cell lines survived only to the third generation.

Several peptones or peptone dialysates have been used as a serum replacement in both medium NCTC 135 (see Serial NCI-3257), NCTC 161, and NCTC 162. Some peptones have also been used in combination with serum albumin, α1 protein or both.

A 15% Witte peptone supplement to NCTC 161 and NCTC 162 (NCTC 161 with 5 mg/liter vitamin E and 0.1% DMSO added) has been quite successful as a supplement thus far. To date one cell line has been carried up to 10 culture generations in medium NCTC 161 with Witte's peptone. One other cell line has gone through 7 subcultures. While these cell lines are presently growing slowly, it is thought that they may be in a "lag" common to freshly explanted cells and will recover and grow continuously soon. Several other cell lines also in medium NCTC 161 supplemented with Witte's peptone are still growing well in the earlier pre-lag state. More recently, 6 cell lines have been initiated in medium NCTC 162 supplemented with Witte's peptone. Thus far their growth has been excellent in low generations, at least for 5 generations.

A bacto-peptone dialysate has also been used as a supplement to NCTC 161. Two cell lines have been grown in this medium without success. Apaprently the bacto-peptone dialysates can only be used as a supplement to already established long-term cell lines as has been demonstrated by other investigators in other laboratories.

3. The influence of low oxygen in establishing and maintaining cells in vitro.

a. Freshly explanted mouse cells: There is published evidence that cells grown in an atmosphere of low oxygen tend to show less chromosome damage than those grown in an atmosphere of air and CO_2. There is also unpublished evidence that cells grown in lower amounts of oxygen tend to clone better. We are trying to determine whether cells initiated and maintained in an atmosphere of low oxygen can be established and maintained as diploid normal cells and/or can be prevented from becoming malignant. To date 12 cell lines have been initiated in an atmosphere containing 1% oxygen, 10% CO_2, and 89% nitrogen. Ordinarily, freshly explanted mouse cells are prepared by mincing with scissors, washing with saline or medium and planting. Most growth comes from the bits of minced tissue planted. We have lear ed that under conditions of low oxygen (i.e 1%) cells do not grow out from the clumps which necrose and die in 1 to 2 days. Recently we have changed our explanting procedure so that more single cells will be planted rather than finely minced pieces of tissue. In this method the finely minced tissue is washed at least 2 times with medium or balanced salt solution in a centrifuge tube. The supernatant is drawn off and between 5 to 10 ml of the growth medium added to the tissue and used to transfer the mince to a regular test tube. The tube is stoppered tightly with silicone stopper and the contents shaken vigorously for several minutes. The tube is then centrifuged for 2 minutes at 2xg and the supernatant withdrawn and used to inoculate the cultures.

Our data thus far indicate that practically single cell suspensions can be obtained by this method and will grow in culture. It also appears that cells prepared in this manner will grow well under conditions of both 20% oxygen and 1% oxygen. Furthermore, preliminary experiments indicate that cells in 1% oxygen grow well in Dulbecco-Vogt medium but poorly in medium NCTC 161. The reason for this difference is being investigated, but this information suggests that use of 1% oxygen may require specific media formulations. In our data it appears that cell suspensions exposed to 1% oxygen tend to adhere to the floor of the flask better than similar cells grown in 10% CO_2 in air. Further studies must be carried out to determine the long-term results of these experiments.

b. Freshly explanted normal human cells: It is generally known that human diploid cells appear to have a finite life and can not be maintained in culture much more than 50 generations. There has been recent evidence that high amounts of oxygen may have an aging effect on human cells. We set out to determine whether by maintaining human diploid cells in an atmosphere of low oxygen content we could extend the life span of human cells in vitro. Thus far we have received human embryos twice but as yet have found the tissue unsatisfactory for in vitro growth. As better tissue is available the question will be reexplored.

c. Long-term diploid normal human cells: Cell line Wi-38 is widely used in studies on normal diploid human cell lines. Exploratory studies are in progress using this line as a prototype to attempt to resolve why human diploid cells have a finite life and to try to reverse this phenomenon by unique culture and nutrition conditions extrapolated and extended from the above studies.

4. Test of growth of C3H mouse cells in large and small molecule fractions of horse and fetal bovine serum.

In three separate experiments over a period of almost 4 years it was determined that mouse embryo cells would grow much better if the large molecule fetal bovine fraction was in the medium. Adding the small molecule fraction of either fetal bovine serum or horse serum did not appreciably improve the ability of this medium for the growth of the cells. The large molecule fraction of horse serum alone or in combination with either the small molecule fraction of horse serum or fetal bovine serum supported limited growth of these cells until the cells ultimately adapted to these media and grew well, after a period of over one year in vitro. Cells in medium NCTC 135 with no supplement or with the small fraction of either horse or fetal bovine serum did not grow continuously as long-term lines, although with the unsupplemented NCTC 135 no assiduous effort was made for characteristic adaptation. These results have been published this year.

5. Capacity of C3H mouse embryo cells to grow in medium containing gelding, mare, or stallion serum from a commercial source.

Three pools of C3H mouse embryo cells were explanted to test stallion, mare, and gelding serums. Two of the pools were from C3H/HeN embryos and the third pool from C3H/He embryos. In all 3 pools growth in NIH gelding horse serum (control serum) was less satisfactory than in commercial gelding, mare, and stallion serum. Of the commercial serums in long-term experiments the best growth has been obtained with the stallion serum. The next best growth was obtained with the mare serum, although the difference in growth potential between it and gelding serum was practically non-significant. The growth of all the cultures in all 4 horse serums was poorer than in fetal bovine serum. In stallion serum, irrespective of the size of initial population, all cells have approximately the same rate of proliferation. The subculture interval was longer in the $C3H_f$/He cells than in the 2 pools of $C3H_f$/HeN cells in the mare and commercial gelding serums. In these 2 serums the size of initial inoculum seems to have an influence; the smallest inoculum appeared to grow more slowly. A manuscript has been accepted for publication.

6. Growth and metabolism of a neoplastic C3H/HeN cell line with emphasis on vitamin B_{12}, folic acid, and nucleic acid precursor interrelationships.

In chemically defined medium containing adenine, deoxyuridine, and 0.57×10^{-6}M folate, but no vitamin B_{12}, the amount of DNA synthesized by a neoplastic C3H/HeN mouse cell strain was comparatively limited. It was increased by substitution of thymidine for deoxyuridine or by addition of B_{12}. When B_{12} was present but the folate level was lowered to 0.57×10^{-7}M and other components were unchanged, less DNA was synthesized; the decrease was partly reversed when deoxyuridine was replaced by thymidine. A further limitation was observed when adenine was left out of the B_{12}-containing medium. Incorporation of radioactivity from both ^{14}C-formate and deoxyuridine-2-^{14}C into DNA-thymidine was limited without B_{12}. Under conditions of culture requiring B_{12} for continuous cell proliferation, the requirement was met by B_{12}, methyl-cobalamin, or hydroxocobalamin at a concentration of 0.7×10^{-6}M. It appeared, however, that methylcobalamin does not provide a pool of methyl groups for DNA-purine or

DNA-thymidine synthesis. That B_{12} functions, in part, to increase DNA-thymidine synthesis in these cells in culture by raising limiting levels of folate coenzymes required in the metabolism of "one-carbon fragments" seems the most probable explanation of these findings. A paper has been published during this year.

7. Test of the influence of different formulations of NCTC media on growth of a human tumor line.

A quasi-diploid cell line derived from an anaplastic squamous cell carcinoma of the nasal septum was used in this study. At the time we obtained these cells they had been maintained in Eagle's minimum essential medium plus 10% fetal calf serum. In this medium they were subcultured every 10 to 14 days. In our laboratory the cells were initially grown in medium NCTC 135 + 10% fetal calf serum. In this medium they have been subcultured every 7 days at a split ratio of 1:3 or 1:4. After one generation in medium NCTC 135 plus fetal calf serum, a daughter line was established in NCTC 161 + 10% fetal calf serum. The cells have grown well in this medium also and can be subcultured every 7 days. In order to determine whether one NCTC medium was better than the other, a quantitative study was set up. Cells grown in each medium were inoculated at 700,000 cells/flask and grown for a period of 10 days in their respective medium. Counts were made at 3 and 10 days with the following results: At the end of 3 days in both media the cells were still in rapid log growth with those in medium NCTC 161 somewhat higher than those in NCTC 135. At the end of 10 days there was twice the population in medium NCTC 161 as in medium 135. This suggests that for one human line, at least, different media can be formulated for specific cell types to yield higher populations within finite periods of time. Such data require confirmation with other human lines over a variety of time periods. Further, this medium NCTC 161 appears to encourage better single cell suspensions from more uniform inoculum populations.

Significance and Proposed Course:

Neither normal nor non-malignant human cells, as well as specific tumor types, whether human or lower animal, are grown as readily in long-term cell culture as are the normal cells of lower animals and fibrosarcomas or highly undifferentiated tumors of lower animals. There are, of course, exceptions to these results. Nutritional factors may have a profound effect on the growth of both normal human and malignant cells. The classic example usually cited is the requirement of leukemic cells for asparagine. In order to have a variety of cells for models, both normal and malignant, numerous formulations of chemically defined media have been evolved. These have been varied in amino acid, salines, vitamins, and other components. Some of these have been employed already for their relative capacity to grow two kinds of human tumors, i.e. Hela and anaplastic squamous cell carcinoma of the nasal septum. Other human strains from different organs, both normal and malignant, are being surveyed for selection of an appropriate battery for further studies.

For some years it has been commonly held that human diploid cells in culture cannot be maintained indefinitely; they either become heteroploid and continue to proliferate or they cease to divide after a finite period in vitro, as with

Wi-38. For the overall cancer plan, long-term growth of normal diploid human cells is paramount for general studies and mass amounts of cells. Effort, therefore, will be made to develop better media and culture techniques in order to obtain this goal. One theory for the reason human diploid cells fail to divide after a finite period in vitro is that they undergo an "aging" process. Studies involving adding large amounts of vitamin E to the culture media are now in progress to determine if this will prolong the life span of human diploid cells. Other studies to determine and isolate specific growth promoting or growth inhibiting substances in serum are also under way. We are also trying to determine whether decreasing the amount of oxygen to which the cells are exposed will increase the finite life of diploid cells, since low oxygen has been found to cause less chromosomal changes in culture and enhance growth of single cells.

The continuing program on nutrition and physico-chemical requirements of cells particularly for advancing studies on differences between nonneoplastic and neoplastic cells still relies on 1) the analytical approach to determine growth promoting factors in known medium supplements such as serum or peptones, and 2) the successful synthetic approach of logical additives to medium formulations of chemically characterized compounds. The leads from the serum fractionation studies suggest investigating the importance of hormone-like substances, unknown vitamin-like materials, polypeptides, and perhaps purines or pyrimidines or their nucleosides. Further simple but time consuming testing of different ratios of vitamins and amino acids may be rewarding for growth stimulation and is being explored. It is also highly desirable to work with autoclavable media to avoid adventitious agents in sera.

Provided a "handle" can be got on culture methodology and nutrition for special types of tumors and the cells from which they are derived, cell, tissue, and organ cultures are extremely important for furthering the research objectives and approaches as proposed in the National Cancer Plan. At the very least, cells, tissues, and organs in vitro are important in objectives 2, 3, 4, 5, and 6. We can cite within these 5 objectives at least 125 project areas (in the few objective and approach books available) where cell, tissue, and organ culture has immediate or correlated or parallel significance or research impact. The activities in our Serial No. NCI-3212, project 1, reported here, we believe, have bearing and will advance the success of the project areas.

Serial No. NCI-3212, Project 2 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project 2, Title: Cell transformations in vitro.

Principal Investigators: V. J. Evans and F. M. Price

Other Investigators: R. R. Gantt, H. A. Kerr, R. A. Dworkin, K. K. Sanford, and S. R. Handleman

Cooperating Units: A. Richter, Laboratory Cell Suppliers, Frederick, Md.; J. W. Hartley, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md.

Man Years: See Serial No. NCI-3211.

Project Description: A study to discover differences between normal and cancerous cells and to determine factors influencing the spontaneous malignant transformation of normal cells to the neoplastic state. National Cancer Plan primarily 3.1 and secondarily 3.2.

Objectives:

1. To study the phenomenon of "spontaneous" malignant transformation of cells in vitro. This type of transformation simulates human cancer.

2. To attempt to determine a factor(s) responsible for this "spontaneous" transformation.

3. To stabilize the tissue culture system so that this "spontaneous malignant transformation does not occur or to delay the time of onset in cells in vitro so that dynamic changes in the non-malignant or pre-cancerous cells to cancer cells may be uncovered; to make systems available so that methods to arrest the event can be explored.

Methods Employed: Experiments were planned to examine the influence of serums on different animal species (horse and bovine) and sex, and of peptones on the spontaneous neoplastic transformation of $C3H_f$ cells in vitro. Cloned populations were obtained to explore the question of whether induction or cell selection is the primary factor in so-called malignant conversion of cells in vitro. Assessment of neoplastic conversion was made by implantation of cells into syngeneic animals and correlations were attempted by evaluation of tumor data and cytologic standards established from nonneoplastic and neoplastic cells.

Major Findings:

1. Comparative effects of mare, stallion, gelding horse serum and fetal bovine serum on neoplastic transformation in vitro.

The objective of this study was to compare the effects of mare, stallion, gelding horse, and fetal bovine serum on the spontaneous neoplastic transformation in vitro of 3 independent pools of C3Hf mouse embryo cells. Presumably these serums would, at least, differ in hormonal content. From earlier studies, it had been determined that such cells in gelding horse serum undergo neoplastic transformation after a relatively short and predictable period in vitro, while in fetal bovine serum the transformation is both markedly delayed and less predictable in time of transformation. In this particular study the gelding horse serum was from a source different from that of the earlier studies. Further attempts were made to diagnose the neoplastic potential of the cells by means of cytologic criteria that have been described and documented for similar cell-culture systems (cf Serial No. NCI-3213). It was found that the 3 pools varied in their potential for neoplastic transformation, with the reason still remaining unclear, although the possibilities exist that unidentified variations in explantation of the original cells or culture conditions including temperature variations or fluctuations in pH might have been influential. Another possibility is that the cells may have become antigenic to the syngeneic host since by cytologic appearance they were neoplastic. It also appeared that mare serum like fetal bovine serum had a delaying or inhibiting effect on spontaneous neoplastic transformation as compared with the 2 male horse serums. Cells in the mare serum showed a prolonged latent period for tumor production. Also, the effect of serum on neoplastic transformation appeared to be unrelated to its growth stimulating capacity since stallion serum and fetal bovine serum were both highly growth stimulating. The results may indicate a sex hormone influence on the spontaneous neoplastic transformation or also that the mare and fetal bovine serums may lack a potentiating factor present in the male horse serums. A manuscript has been submitted on this study.

2. Effect of fractions of horse serum and fetal calf serum on neoplastic transformation of C3H mouse cells in vitro.

Some time ago we found that C3H/He and C3H/HeN mouse embryo cells when cultured in horse serum underwent neoplastic transformation after a comparatively short period in vitro and at a time that subsequently was revealed to be relatively predictable. This transformation in sister cultures cultured in fetal bovine serum was not as predictable and, if it occurred, did so after a much longer interval in vitro. The cells in horse serum grow less rapidly, certainly initially, and show chromosomal change earlier than cells in fetal bovine serum. We have subsequently cultured C3H cells in horse serum, fetal bovine serum, or various combinations of large and small molecule fractions of horse and fetal bovine in chemically defined medium NCTC 135. Cells grown in NCTC 135 alone or with the small molecule fraction of either horse or fetal bovine serum did not grow as continuous long-term lines. The best growth was obtained when the cultures were in a medium containing the large molecule fraction of fetal bovine serum either alone or in combination with a small molecule fraction. Cells grown in the presence of the low molecular

weight (5000) fraction of horse serum (for as short a period as 200 days) invariably produced tumors on injection into syngeneic animals. Cells in the small molecular weight fraction of fetal bovine serum combined with the large molecular weight fraction of horse serum produced tumors only after a period in vitro, i.e., 784 days. Two simple and plausible rationales are apparent. First, the ultrafiltrates from both horse serum and fetal bovine serum contain a factor(s) which converts the cells. The horse serum might contain a higher concentration or a more potent agent. A second and equally simple alternative is that the horse serum ultrafiltrate contains the active factor(s) but that a small amount remained with the large horse serum fraction perhaps tightly bound. The data on ultimate conversion with whole fetal bovine serum gave great credence to the concept that the converting agent is in fetal bovine serum and horse serum, but the effective concentration or potency is measurably greater in horse serum. Argument can be marshalled for some protective effect of the large molecule fractions of serums, particularly since cells in serum-free medium can, after very long periods, undergo malignant transformation.

3. Influence of peptones in growth medium on neoplastic transformation.

The need to substitute for serums, growth promoting factors which do not introduce agents that pass through micropore filter is great. Various autoclavable peptones have been employed as such serum substitutes for the growth of cells. Their possible influence on malignant conversion of mouse cells is under test, since mouse cells in both horse serum and calf serum (cf Serial No. NCI-3213) have been found to be converted to neoplastic cells. Two sublines in Witte's peptone, an enzymatic digest of beef fibrin, are being assayed in animals. These media have required to date additional supplementation of 3x crystallized albumin. The animals will be held for 1 year for precise and definitive assurance of neoplastic potential of cells. Other lines in peptones with no other additives and with improved chemically defined media have not yet produced populations adequate for animal assay. They are currently undergoing the characteristic growth lag shown by cells explanted in vitro. This study is part of an ongoing endeavor to stabilize nonneoplastic cells in vitro.

4. Efforts to distinguish between induction and cell selection as they influence malignant conversion of cells in vitro.

Answers to the question of whether induction, cell selection, or both, operate to influence the so-called spontaneous malignant conversion of cells in vitro are dependent upon establishment of clonal derivatives from strains of cells that are non-malignant. Recently through the purchase order system, one clone selected by microplating has been obtained from a strain cultured in fetal bovine serum and has not produced tumors. Others are in preparation. This clone was obtained employing the system of exposing the cells to reduced oxygen which appears to increase plating efficiency (see Serial NCI-3213). The clone has been built up to numerous cultures in fetal bovine serum. Six sublines have been transferred to either stallion horse serum millipore filtered, or gelding horse serum for conversion (see Serial NCI-3212 project 2). Baseline injections to C3H mice of the parent clone in fetal bovine have been made over 4 months ago and the clone still seems to be non-malignant. The

derivatives exposed to the two types of horse serum for conversion which usually occurs in mixed populations only after four plus months exposure have not yet been exposed for sufficient periods for current injections with concomitant latent period delays to be meaningful. At the time of injections cytological assessment and karyology for correlation with injection data will be obtained. From the line in fetal bovine serum and sublines in horse serum the growth rates can be determined by microcinematography as a single example of a possible difference between malignant and non-malignant cells.

Significance and Proposed Course:

The data on effects on male, female, and gelding horse serum and of large and small molecule fractions of horse serum (gelding) and fetal bovine serum upon the malignant or neoplastic conversion of cells raise several questions. We are initiating experiments to determine if cells can be maintained indefinitely without neoplastic conversion or at least select a serum fraction which will be more likely to extend the in vitro period in which the cells are not neoplastically transformed. By carrying out appropriate experiments on both fractions of serum from both sexes, we will attempt to determine definitively if the neoplastic transforming effect of small molecule horse serum can be confirmed unequivocally and if it is sex related. Again, with appropriate fractions, we will determine if there is a suppressive effect on the malignant transformation from large molecule female horse serum or large molecule fetal bovine serum. Preparation of all these fractions is a laborious technical chore requiring several months in order to obtain the mass amounts of material required for the long-term studies required to answer the questions posed and to gain some explanation for the reason of so-called "spontaneous" neoplastic conversion of cells in vitro. Regrettably, commercial sources of the desired serum fractions for these studies are not available. The inability to stabilize cells as non-malignant for indefinite periods hinders the effective use of cells in vitro for multiple ancillary studies so it is highly important to resolve the factors responsible for the transformation.

For some time the quality of serums has been suspect. Serum is often collected under adverse conditions and may be contaminated. Occasionally serums contain viruses or mycoplasma, which are not easily detected or removed. Also, serums may vary in protein, hormone, trace metal, etc., content from species to species or from lot to lot within the same species. It therefore has become necessary to find a replacement for whole serum which will promote growth of cells as well as serum, but is more defined in chemical nature and can be obtained easily. Ideally, a completely defined growth-promoting factor should be used. To this end we are investigating the use of peptones and/or substances derived from them. We shall continue studies in progress to determine whether peptones can be used as a supplement to growth media in place of serums. Further studies will encompass isolation and purification of definite substances from peptones which will supplement the media. (See also Serial No. NCI-3257.) In this way it will be possible to produce uniform media to be used in studies involving "spontaneous" neoplastic transformation of mammalian cells.

To resolve the question of whether the serum effect is an induction phenomenon or the result of cell selection, establishment of clonal derivatives

from non-malignant cells is required. From a recently isolated non-malignant clone, some sublines have been continued in fetal bovine serum while others have been cultured in gelding horse or stallion serum. The data from ongoing injections of these lines in the 3 serums should tell us whether, in the case of cells in the fetal bovine serum, this particular serum has operated in the earlier studies on mixed populations to select cells of a type unlikely to undergo malignant transformation or whether the fetal bovine serum stabilizes cells in a state more nearly approaching normality. Should the cells from this clone when subjected to horse serum become transformed, then one would infer that the horse serum operates to induce a transformation, since selection has been ruled out by use of derivatives of a single cell. If the cells do not transform, then one must assume that the earlier noted effect of horse serum in mixed populations has been one in which the horse serum selected cells more likely to be transformed. In addition, exposure of the cells to either stallion or gelding serum followed by subsequent injection of the cells should if transformed tell us the relative efficacy of these 2 serums in transforming the cells and indicate where a greater pool of the transforming agent(s) is likely to be found. The possibility also exists that we may uncover as an additional bonus a desirable cell model for rapid conversion of cells in vitro more useful than the known viral or chemical carcinogens which can in some cases be prolonged.

The obvious usefulness of characterized non-malignant and malignant cell lines from a single cell in many projects is far reaching, particularly for comparative biochemical studies.

The significance of these studies is receiving new emphasis in the light of the National Cancer Plan. Innumerable approach elements and project areas are requiring cell lines under stable conditions to study effectively such things as surface differences between normal, pre-cancerous, and cancerous cells (2.4.3.7) the influence of environmental and genetic factors on development of malignancy (3.1.1.6) with the ultimate objective of evolving rational methods of preventing conversion to malignant cells, the significance of DNA repair on reversion of malignant cells, the role of interferon on conversion of normal cells to malignant cells (3.1.1.12), and the significance of maintaining differentiation to prevent transition of normal cells to malignant cells, and for controlled models for all studies on carcinogenic agents.

Honors, Awards, and Other Activities (Dr. V. J. Evans)

1. Vice President, Tissue Culture Association, 1968-1970, 1970-1972.

2. Candidate for President, Tissue Culture Association, 1972-1974.

3. Member, Board of Governors, W. Alton Jones Cell Science Center, Lake Placid, New York, 1970-1972.

4. Chairman, Executive Committee, Board of Governors, W. Alton Jones Cell Science Center, Lake Placid, New York, 1970-1972.

5. Member, Committee to Select Additional Members, Board of Governors, W. Alton Jones Cell Science Center, 1970-1972.

6. Chairman, Insurance Committee, W. Alton Jones Cell Science Center, 1970-1972.

7. Member, Endowment and Funding Committee, W. Alton Jones Cell Science Center, 1970-1972.

8. Consultant for Index of Tissue Culture, prepared by National Library of Medicine and published by October House; literature in field from 1966-1969 and for current new volume.

9. Consultant to Interagency Board, U. S. Civil Service Commission.

10. Member, Editorial Board, Journal of Cryobiology, to annual year 1972.

11. Invitation to prepare section on Replicate Culture Methods, 1. Monolayer Cultures, in Methods and Applications of Tissue Culture, editors P. F. Kruse and M. K. Patterson, Academic Press, Inc., New York, 1972.

12. Consultant Ex Officio to Education Committee, Tissue Culture Association.

13. Consultant Ex Officio to Constitution Committee, Tissue Culture Association.

Publications

1. Price, F.M., Gantt, R.R., Evans, V.J.: Effect of fractions of horse and fetal bovine serum on neoplastic conversion of C3H mouse cells in tissue culture. In Vitro 6: 437-440, 1971.

2. Rotherham, J., Price, F.M., Otani, T.T., Evans, V.J.: Some aspects of the role of vitamin B12 and folic acid in DNA-thymidine synthesis in a neoplastic C3H mouse cell strain. J Nat Cancer Inst 47: 277-287, 1971.

3. Taylor, W.G., Price, F.M., Dworkin, R.A., Evans, V.J.: A simple device for planting replicate mammalian cell cultures. In Vitro 7: 1972.

Serial No. NCI-3213, Project 1 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project 1, Title: Carcinogenesis in vitro.

Principal Investigator: K. K. Sanford

Other Investigators: S. L. Handleman, M. L. Hursey, C. U. Garrison, E. V. Peppers, H. A. Kerr, V. J. Evans, R. R. Gantt, W. G. Taylor

Cooperating Units: R. Parshad, Department of Pathology, Howard University School of Medicine, Washington, D. C.; J. W. Hartley, Laboratory of Viral Diseases, NIAID, NIH; A. Richter, Laboratory Cell Suppliers, Frederick, Md.

Project Description:

Objectives:

1. To elucidate the cause(s) of "spontaneous" neoplastic transformation of mammalian tissue cells in vitro.

2. To understand the serum influence on spontaneous neoplastic transformation.

3. To examine the effects of a chemical carcinogen on skin cells from normal humans and those with genetic defects and high cancer risk.

4. To improve plating efficiency and clonal growth of mammalian tissue cells for carcinogenesis studies.

Methods Employed: General methods employed will be described under each study.

Study 1.--Possible causes of "spontaneous" neoplastic transformation of mammalian tissue cells in vitro.

A. Endogenous murine leukemia virus (MuLV) as a possible direct or indirect cause of neoplastic transformation in mouse cells.

The hypothesis of a viral etiology of spontaneous neoplastic transformation in vitro would be strengthened if a correlation could be established between presence or activation of MuLV and onset of neoplastic transformation. In the present search for such a correlation, cell lines from 3 pools of normal C3Hf mouse embryo cells were examined during long-term culture for both "spontaneous" neoplastic transformation and presence of MuLV group-specific antigens or infectious virus. Tests for neoplastic transformation were made by implantation of cells into syngeneic hosts and examination for sarcomas at the

injection site. Cells were also examined cytologically for evidence of neoplastic transformation. No correlation between presence of antigen or infectious virus and neoplastic transformation could be established among the 48 cell lines studied.

If MuLV is the direct cause, one might expect that a larger proportion of cell lines with MuLV gs antigen would have undergone neoplastic transformation and that a larger proportion of lines that had undergone neoplastic transformation would be gs antigen positive. Also, spontaneous activation of MuLV appeared to have occurred in certain cell lines without evidence of neoplastic transformation thereafter. The results support the concept that MuLV is rather widely distributed in embryo cells of the $C3H_f$ mouse but is probably not a single direct cause of spontaneous neoplastic transformation in vitro.

A manuscript on this study is ready to be submitted for publication, entitled "Spontaneous" neoplastic transformation in vitro: Influence of endogenous murine leukemia virus and serum fractions.

B. Chromosomal aberrations.

In the many published investigations on the temporal relationship between chromosomal aberrations and neoplastic transformation in vitro, little definitive evidence of causal relationship between the two phenomena has been obtained. However, in almost all cases, chromosomal aberrations precede neoplastic transformation by variable intervals. A line of rat cells was found to retain 80% diploid cells with no qualitative changes in chromosomes and to grow as tumors. The tumor cells are currently under investigation to establish whether spontaneous neoplastic transformation can occur without prior chromosomal alterations. Similar analyses on clones of tumorigenic Chinese hamster cells are in progress.

Factors influencing the stability of chromosomes in mouse cells are also under investigation.

Chromosomes of a line of cells derived from $C3H_f$ mouse embryos were studied after the cells had been exposed to continuous intermittent gassing with humidified gas mixtures containing 20 and 2% oxygen. Cells supplied with 20% oxygen underwent a more rapid shift from diploidy and had a higher frequency of abnormal chromosomes than those grown at 2% oxygen. The results suggest that the oxygen tension of the gas environment may be an important factor influencing karyotypic stability in vitro.

Since the chromosomes of inbred mice are much less stable than those of non-inbred animals such as man, pig, and Syrian hamster, we are currently comparing the chromosomal stability of wild mouse tissue cells as compared with those from an inbred mouse to establish whether long-continued inbreeding of mouse stocks has led to increased susceptibility to chromosomal breaks in cultured cells.

C. DNA damage and impaired DNA repair.

The type of serum used to supplement chemically defined medium was shown previously to influence the time of neoplastic transformation of certain

cells in vitro. In horse serum supplement, C3H mouse embryo cells underwent early neoplastic conversion as compared with fetal bovine serum supplement. Horse serum also resulted in a higher initial rate of chromosomal alterations. Experiments are in progress to establish whether horse serum as compared with fetal bovine serum may impair DNA repair. From our studies to date, mouse embryo cells show a very high rate of ^{3}H-thymidine uptake in both control and UV-irradiated cells. This rate is still high in cells treated with hydroxyurea to suppress semi-conservative DNA replication. By combining microcinematography for mitotic rates and autoradiography, we are finding what appears to be a high rate of unscheduled DNA synthesis in cultures after a short period of culture in vitro in either horse serum or fetal bovine serum. Thus, DNA breaks and repair are occurring in a large proportion of the cells. By these methods, we may be able to 1) identify factors in the culture environment that are causing DNA breaks, 2) establish whether horse serum impairs DNA repair, and 3) whether there is any causal relationship between DNA damage and neoplastic transformation.

Study 2.--The serum influence on neoplastic transformation.

A. Mechanism of the transformation.

One important question that has never been resolved is whether the serum effect on neoplastic transformation (described in 1-C above) results from selection of cells or from cell change. After several years of effort, we have obtained this year a clone from a non-neoplastic culture. This clone has been carried in triplicate in fetal bovine serum and stallion horse serum supplement to establish whether horse serum induces neoplastic transformation. This study is in progress, and we are attempting to obtain a second clone from recently isolated tissue cells.

B. Chemical analyses of serums influencing neoplastic transformation.

Three types of serum that have been found to influence the "spontaneous" neoplastic transformation of mouse cells in vitro were analyzed for certain constituents. The choice of constituents was made on the basis of hypotheses proposed to explain the serum effect. Because the small molecular fraction of gelding horse serum appears to accelerate neoplastic conversion, and in view of the conditions for maintenance of the horses, the horse serum was tested for possible contamination with small molecular chlorinated hydrocarbon insecticides and lead or mercury. Contamination was negligible. Fetal bovine serum, which appears to inhibit neoplastic transformation, was compared with horse and calf serum with respect to certain hormones, 2 enzymes, and 16 elements. Fetal bovine serum differed from the other serums particularly in having an increased level of zinc and high activities of the enzymes hexosaminidase and galactosidase, as well as slightly higher levels of cortisol, iron, potassium, phosphorus, and magnesium, with a lower amount of copper. Heating the serums to 56°C for 30 minutes appeared not to destroy the factors influencing neoplastic transformation. The analyses of this study tended to eliminate at least 4 of the 5 hypotheses proposed to explain the serum effect. This study has been submitted for publication.

C. Influence of fetuin and non-fetuin fractions of fetal bovine serum.

The effects of various fractions of fetal bovine serum on neoplastic transformation were explored. Under the conditions of the experiments, no one fraction consistently delayed or accelerated neoplastic transformation as compared with whole unfractionated fetal bovine serum.

The results of this study are included in the manuscript referred to in Study 1A.

Study 3.--Carcinogen effects on human skin cells in culture.

Studies on hamster and mouse cells treated with different chemical carcinogens and described in previous annual reports are being prepared for publication. In addition, as a collaborative project with Dr. Parshad, cell lines have been initiated from skin biopsies of normal humans and those with genetic defects associated with high cancer risk, i.e., Xeroderma pigmentosum, Fanconi's anemia, Basal cell nevus syndrome, Down's syndrome, and Ataxia telangiectasia. Each of these lines has been carried for 6 weeks with and without the chemical carcinogen benzo(a)pyrene.

The objective of this study is to examine the influence of genetic factors and carcinogen treatment on 1) finite life time in vitro, 2) cytologic manifestations indicative of neoplastic transformation as established for rodent cell lines, 3) chromosomal changes, and 4) DNA repair. As noted in Project 2, we hope to develop some criteria for neoplastic transformation in human cells in vitro.

Study 4.--Factors influencing plating efficiency and single cell growth in vitro.

Clones from single cells taken directly from normal tissues or from cultures of nonneoplastic cells, as well as more effective plating techniques, are needed for studies on carcinogenesis in vitro. In an effort to develop more nearly optimal conditions for the growth of single nonneoplastic tissue cells, we have examined the influence of certain physico-chemical factors on the plating efficiency of embryo cells from freshly isolated tissues and from short- and long-term culture. Cells from the mouse, hamster, and rat were used. The major finding is that growth is always equal or better at 1-3% oxygen than at 18 to 20% oxygen as found in atmospheric air. The reducing agents cysteine and thioglycollate did not enhance plating efficiency. Certain culture media increased plating efficiency. The results of this study and an interpretation of possible mechanisms are being prepared for publication.

Proposed Course: Studies 1B, 1C, 2A, and 3 will be continued to attain the objectives enumerated.

Honors, Awards, and Other Activities:

Member, Advisory Committee of the Animal Cell Culture Collection of the American Type Culture Collection
Member, Editorial Advisory Board of Cancer Research
Member, Editorial Board for In Vitro
Assistant Editor for In Vitro

Publications:

1. Parshad, R. and Sanford, K.K.: Oxygen supply and stability of chromosomes in mouse embryo cells in vitro. J Nat Cancer Inst 47: 1033-1035, 1971

2. Sanford, K. K.: "Spontaneous" neoplastic transformation in vitro. Acta Unio Internat Contra Cancrum. In press.

3. Sanford, K. K., Jackson, J. L., Parshad, R., and Gantt, R. R.: Some evidence for an inhibiting influence of fetal bovine serum on "spontaneous" neoplastic transformation in vitro. J Nat Cancer Inst. In press.

4. Evans, V. J., Price, F. M., Sanford, K. K., Kerr, H. A., Handleman, S. L.: Comparative effects of mare, stallion, gelding horse and fetal bovine serums on neoplastic transformation in vitro. J Nat Cancer Inst. In press.

Serial No. NCI-3213, Project 2 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project 2, Title: Manifestations of neoplastic transformation in vitro.

Principal Investigator: K. K. Sanford

Other Investigators: S. L. Handleman, M. L. Hursey, C. U. Garrison, H. A. Kerr.

Cooperating Units: C. C. Ting, General Laboratories and Clinics, NCI

Project Description:

Objective: To identify any characteristics of cells in culture that may be directly or indirectly associated with their neoplastic transformation in vitro

Study 1: Cytologic characteristics: A comparative study of cytologic changes in cultures of embryo (fetal) cells of human, rat, hamster, and mouse origin during long-term culture.

The major objective is to establish more quantitative, objective cytologic criteria for neoplastic transformation of cells in vitro and a systematic approach to in vitro diagnosis of neoplastic state. Minor objectives are: 1) to attempt to apply criteria established and validated for rodent cells to human cells; 2) to compare responses of cells in a new chemically defined medium with horse serum or fetal bovine serum supplement with earlier results with serum-supplemented medium NCTC 135; 3) to determine whether human cells can be established as permanent long-term lines in the new medium formulation.

The following pools of cells were used to initiate cell lines: 3 pools of 13-day C3Hf mouse embryo cells, 3 pools of 18-day Fisher rat embryo cells, 3 pools of 14-day Syrian hamster embryo cells, 2 pools of human embryo cells. The derivative lines were grown on an improved MEM formulation, supplemented with either horse serum or fetal bovine serum. A permanent record of cytologic characteristics in the form of stained coverslips has been prepared. The rodent cells have been assayed in vivo for neoplastic transformation. The human cells have been examined for changes in enzyme isozymes.

In results to date, several of the human lines have exhibited a finite life time and none of these have shown cytologic evidence of neoplastic conversion. Neoplastic conversions in the new medium have occurred in two mouse lines and one rat line. These results and the tumor latent periods correlated with the results of cytologic diagnosis, i.e., high scores (indicating cytologic changes) correlated with short tumor latent periods and low scores with long latent periods.

Additional data are required from the injections before a definite conclusion can be made concerning the success of a systematic key to cytologic characteristics in predicting and diagnosing neoplastic transformation in vitro.

Study 2: Fetal antigens in cells undergoing neoplastic transformation in vitro. Collaboration with Dr. Ting.

Objective: To study the expression of fetal antigens in cultured cells from the C3H mouse during early culture, late culture before neoplastic transformation, and late culture after neoplastic transformation.

The isotopic antiglobulin technique for measuring cell surface antigens is used. The dilution titration and adsorption techniques are used to look for antigen specificities.

In results to date, mouse fetal tissue in early culture has more fetal antigens than cells in late culture after neoplastic conversion. Efforts are being made to standardize the test cells and preserve these by freezing. In vivo experiments will be carried out to study the immunogenicity or immunosensitivity of the fetal antigens in these cells. The fetal antigens in various tumor cells will be studied to examine their specificities relative to the C3H mouse cells at various passages before or after neoplastic transformation.

Proposed Course: These two studies will be continued to attain the objectives listed.

Serial No. NCI-3234A SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Primary biochemical alterations leading to neoplastic conversion.

Principal Investigator: R. R. Gantt

Other Investigators: V. J. Evans, K. K. Sanford, B. T. Julian, G. H. Smith, and K. J. Stromberg

Project Description: A program to elucidate the primary chemical alterations which are necessary biochemical changes leading to neoplastic transformation.

Objectives:

To apply the various biochemical techniques to a system developed in the Tissue Culture Section (see NCI-3211) in order to identify and understand the chemical events leading to neoplastic transformation. There are three immediate objectives:

1. To identify primary changes in the methylation of nucleic acids during neoplastic transformation and other basic cellular alterations such as pre-neoplastic changes and viral infection.

2. To identify the causal agent (or agents) responsible for the dramatic difference between tissue culture media containing fetal bovine serum and media containing horse serum (see NCI-3212 and NCI-3213).

3. To compare in a general way the physical and chemical difference in the membrane components of some RNA tumor viruses and their host cells.

Methods Employed:

1. In order to identify primary changes in the methylation of nucleic acids, significant differences are first being established before and after neoplastic conversion. These differences will then be correlated with conversion time and with the method of conversion (i.e., viruses, chemical carcinogens, or the horse serum-fetal bovine serum system). These differences are monitored by double isotope co-elution chromatography and enzyme analysis.

2. Identification of the agent (or agents) responsible for the difference in fetal bovine serum and horse serum is being carried out by a series of parallel fractionations and appropriate assay of these fractions. This consists of ultrafiltration through a collodion membrane, Sephadex gel filtration,

ammonium sulfate precipitation, and subsequent tests for their ability to transform cells or to protect the cells from transformation.

3. The discovery of N^2-guanine RNA methyltransferase in AMV has suggested that other oncogenic RNA viruses may have the same or similar activities associated with the virion. Therefore, available viruses have been examined for RNA methylase activities.

Major Findings:

1. Studies utilizing the paired neoplastic and nonneoplastic cell lines have been delayed because of the temporary diversion of resources to complete the studies concerning the methylase enzymes associated with oncogenic viruses. They will be re-initiated during the next fiscal year.

2. A large scale fractionation of fetal bovine, mare, and stallion sera has been completed for a two to three year experiment designed to elucidate the differences between these sera with regard to their relative abilities to delay or accelerate malignant conversion. Results from this work will have to await completion of this long-term experiment.

3. Results demonstrating RNA methyltransferase enzymes associated with RNA tumor viruses seem to be forming a pattern. The leukemia viruses appear to have only N^2-guanine RNA methyltransferase activity (Gantt, Stromberg, Montes de Oca, 1971; Gantt, Smith, Julian, 1972), while the sarcoma viruses seem to have both N^2-guanine and a 6-methyl-amino purine RNA methyltransferase activity. Also, the activity is apparently not in the core of the virion, suggesting that a nucleic acid component in the host cell is the natural methyl group acceptor rather than the nucleic acid associated with the virus (Gantt, Stromberg, Julian, 1972).

Significance and Proposed Course of Investigation:

1. Upon completion of the first phase of the virus-associated methylase work the paired neoplastic and nonneoplastic cell lines will be used to focus on the relationship of "spontaneous" malignant conversion and the two specific methylase enzymes found in oncogenic C type viruses. This approach is strongly suggested by a previous finding (Quinn, Gantt, Evans: Comparison of methyl-labeled tRNA in a neoplastic cell line with a paired nonneoplastic control. Exptl & Molec. Pathol. 13: 231, 1970) in which a difference in methylation was found between the neoplastic cell line and its nonneoplastic control where the hydrolysis data indicated the difference resided in a methylated purine. The significance of this is that both the viral induced conversion and the "spontaneous" conversion may both be a result of a common aberrant methylation event.

2. A full discussion of the serum work appears in the following reports: See Serial No. NCI-3212 and NCI-3213.

3. The discovery of a specific RNA methylase associated with all the C-type viruses examined to date is very important in view of the large body of evidence implicating aberrant methylation with malignant conversion. The

proposed course of investigation is as follows:

1. Purify and characterize the enzyme for two reasons: first, to produce antibodies so that single cell assays can be developed for its presence; and second, establish the neighboring nucleotide specificity to assess whether the enzyme, though present, is actually active because of the known presence of specific inhibitors.

2. Assess the role of the specific methylase in the virus life cycle.

3. Look for the specific presence of N^2-methylguanine in the paired neoplastic and nonneoplastic cell lines.

4. Establish the relationship and significance of the 6-methylaminopurine activity found with the sarcoma viruses to the sarcoma virus life cycle.

Publications:

1. Gantt, R. R.: In vitro tRNA methylation of paired neoplastic and nonneoplastic cell cultures. Cancer Res. 31: 609-612, 1971.

2. Gantt, R. R., Stromberg, K. J., and Montes de Oca, F.: Specific RNA methylase associated with avian myeloblastosis virus. Nature 234: 35-37, 1971.

3. Price, F. M., Gantt, R. R., and Evans, V. J.: Effect of fractions of horse and fetal bovine serum on neoplastic conversion of C3H mouse cells in tissue culture. In Vitro 6: 437-440, 1971.

4. Gantt, R. R., Stromberg, K., and Julian B.: Absence of RNA methylase in the AMV core. Accepted, J. Virol., 1972.

5. Sanford, K., Jackson, J., Parshad, R., and Gantt, R.: Some evidence for an inhibiting influence of fetal bovine serum on spontaneous neoplastic transformation in vitro. Accepted, J. Nat. Cancer Inst., 1972.

6. Gantt, R., Montes de Oca, F., and Evans, V. J.: Methylation of satellite DNA in mouse neoplastic and nonneoplastic cell cultures. Submitted to Biochim. Biophys. Acta., 1972.

7. Gantt, R., Smith, G., and Julian, B.: Base specific methylase activity in RNA tumor viruses. I. Avian leukosis virion-associated RNA methylase(s). Submitted to Virology, 1972.

Serial No. NCI-3257 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Factors influencing the growth and neoplastic conversion of cells in vitro.
(National Cancer Plan Approach 2.4, Project Area 2.4.3.3)

Principal Investigator: W. G. Taylor

Other Investigators: V. J. Evans, K. K. Sanford, R. A. Dworkin, F. M. Price, E. V. Peppers, and M. J. Taylor.

Project Description:

1. Design and validation of a replicate culture planting device for nutritional and chemical carcinogenesis studies in vitro.

Objective: To modify currently available laboratory equipment so that large numbers of biologically and statistically identical (replicate) tissue cultures could be prepared rapidly and simply with a minimum of cell damage.

Methods Employed:

A cap for the mouth of a Florence flask was made from a silicone stopper. A glass tube for withdrawing aliquots and a gas (10% CO_2, 90% air) supply and exhaust ports were inserted through the cap. The sample withdrawal tube was connected to a Cornwall automatic syringe pipette, and the entire unit autoclaved before use. A cell suspension was aseptically prepared in the Florence flask and stirred in growth medium with two 15x1.5 mm magnetic stirring bars. Samples were dispensed by aspirating the Cornwall pipette. Statistical and pH variation from sample to sample was analyzed by resuspending NCTC 2071 (strain L grown continuously in chemically defined medium) in medium NCTC 135 and dispensing a minimum of 60 samples per evaluation in 3 different aliquot sizes. The pH of the 30 odd numbered samples was evaluated immediately by comparison with a LaMotte phenol red indicator set; the 30 even numbered samples were brought to a final volume of 10.0 ml and counted on a Coulter Model B counter.

Cell growth following replicate culture preparation was evaluated with 5 day old parent cultures of clone L-2071 in fully defined NCTC 135 (Evans, V.J., et al., Exptl. Cell Res. 36: 439-474, 1964), with Merchant's L-M cells (CCL 1.2) (Hsu, T.C. and Merchant, D.J., J. Nat. Cancer Inst. 26: 1075-1083, 1961), with a rabbit myocardium line obtained from Dr. Robert W. Pumper and continuously propagated in MEM plus Bacto-peptone dextrose (Pumper, et al., Nature, 207: 662-663, 1965), and with rat embryo strain in the third passage level in Dulbecco-Vogt's modified MEM plus 10% FBS. Monodispersed cell pools of clone L-2071, L-M, and rabbit heart were prepared by disrupting the cell

sheet with a cellophane mop while the serum supplemented rat strain was trypsinized, and each resuspended at a known population density. Aliquots of 3 ml were dispersed to several T-15 flasks using the replicate culture planting unit. After a predetermined incubation period, the cell population in each flask was harvested and counted on a Coulter Model B counter.

Major Findings:

The greatest variation in cell number from sample to sample was observed with 0.5 ml aliquots dispensed with a replicate cell culture planting unit incorporating a 2 ml Cornwall syringe pipette. The percent deviation was less for 1.0 ml and 3.0 ml samples delivered from units consisting in part of a 2 ml and 5 ml Cornwall syringe pipette, respectively. Inherent in these cata is any error introduced by the Coulter counter itself. The pH of the planting medium remained, for all intents and purposes, constant throughout the 30 minute period required per evaluation, dropping less than 0.1 pH unit during the first few minutes, then stabilizing for the duration of the test.

Strain L (NCTC 2071), rabbit heart, L-M, and the rat embryo cells were dispersed in culture medium, planted in the usual way, and incubated for the prescribed time. In each case, the resulting populations appeared healthy and grew as anticipated, indicating no deleterious effect from the stirring of the suspension or from the method of dispensing aliquots.

Significance:

Through its design, the replicate culture planting unit permits continuous gassing of mammalian cell suspensions with humidified CO_2/air, thus stabilizing the pH of sodium bicarbonate buffered culture medium ($\pm$ <0.05 pH units). The unit can be readily varied to deliver different volumes, and cell samples can be dispensed simply and rapidly with minimum cell damage or chance of microbial contamination. Quantitative data demonstrate that cell populations planted with this device are relatively uniform. This unit has been used with good success in several studies in this laboratory, including studies on the influence of reducing agents in vitro, chemical carcinogens, and the nutritional studies described below.

2. Efficacy of commercially available peptones as cell growth stimulants and/or substitutes for serum.

Objective: Bacto-peptone (Difco, Inc.) is used widely as either a supplement in serum containing culture medium, or as a substitute for serum in chemically defined medium. The purpose of this investigation was to evaluate other commercially available peptones for their biological efficacy in vitro.

Method Employed:

Peptones from several sources were reconstituted in either a) Earle saline (filter sterilized), or b) triple glass distilled water (autoclave sterilized). The peptones were added to the growth medium used either (1) on a biuret basis wherein a biuret analysis was performed on each stock peptone and the volume of peptone employed/100 ml growth medium adjusted to be equivalent to the biuret response of fetal bovine serum (4.2 gm %), or (2) on a 0.5-1% weight of peptone

per volume of culture medium basis. Cell lines used included: NCTC 2071 continuously grown in protein-free NCTC 135; Merchant's L-M cell line continuously grown in 0.5% w/v Bacto-peptone supplemented medium 199; and Pumper's rabbit heart (RH) cell continuously subcultured in 1.0% w/v Bacto-peptone supplemented MEM, 199 (Morgan, J.F. et al., Proc. Soc. Exptl. Biol. Med. 73: 1-8, 1950), MB752/1 (Waymouth, C., J. Nat. Cancer Inst. 22: 1003, 1956), and NCTC 135.

Cell cultures were planted at 1-1.5x10^5 cells/T-15 flask in 3 ml of unsupplemented medium with the replicate culture planting unit (see section 1 above), and after an incubation period sufficient to allow greater than 99% of the cells to attach to the floor of the flask, the planting medium was removed and replaced with fresh experimental medium. Cultures were incubated 5 days in the various experimental media, at which time they were harvested (with 0.25% trypsin) and counted on a Coulter Model B counter. Data were expressed as the fold increase in cell number, that is, the ratio of the final cell number per flask to the original seed inoculum.

Major Findings:

Preliminary studies indicated that 3 of the original 9 peptones tested were unsuitable for tissue culture purposes, and that the autoclaved preparations were more efficacious than the filtered fluids. Also, the growth of NCTC 2071 was greatest in unsupplemented NCTC 135. Due to this, it was concluded that this cell line was a poor model for further studies, and all subsequent experiments were performed with the L-M or RH cell lines.

From the subsequent studies, it was concluded that Peptic Peptone (NBCo), a peptic digest of beef lung, was as efficacious as the more commonly employed Bacto-peptone (Difco, Inc.) for cell lines adapted to continuous growth in peptone-supplemented growth medium. Witte's peptone was found to have marginal activity, and has been used successfully for other related studies (see section 3).

Significance and Proposed Course of Study:

The use of a chemically defined culture medium supplemented with serum or other biologics is a common, expedient, but less than satisfactory practice. Serum is known to contain enzymes, hormones, "natural" antibodies, and cytotoxic factors; it can be the source of contaminants such as mycoplasma and adventitious viruses. Moreover, the present lack of large volumes of high quality, standardized serums emphasizes the need for a biologically efficacious replacement. Paradoxically, most if not all currently available diploid cell strains require serum for their growth and survival, and the cultivation of established lines is more difficult in its absence. The isolation and characterization of the serum component(s) responsible for its in vitro activity is a highly complicated and incomplete task, and when attempted the fractions, unlike those of Bacto-peptone, fail to support cell growth to the same degree as whole serum. Most importantly, it has been repeatedly demonstrated that the type and quality of serum used profoundly influences the malignant conversion (transformation) of cells in vitro.

Peptones represent one potential substitute for serum in tissue culture medium. Ongoing and future work includes the use of peptones for initiating primary mouse cell cultures (see section 3), the purification of whole peptones so as to isolate the biologically efficacious component(s) (see section 4), and the application of the results of these studies to human cell culture systems, particularly with respect to the formulation of an "enrichment" medium for human cancer cells.

3. Use of commercially available peptones, crystaline albumin, and α_1-globulin as whole serum substitutes for primary C3H/HeN embryo cell cultures.

Objective: Todaro and Green (Proc. Soc. Exptl. Biol. & Med. 116: 688-692, 1964) and Matsuya and Yamane (Proc. Soc. Exptl. Biol Med. 135: 893-898, 1970); Tohoku (J. Exptl. Med. 101: 379-385, 387-396, 1970) have reported the successful use of albumin for primary cultures of a variety of species of tissue culture cells. Holmes has reported that α_1-globulin is efficacious for a variety of human cell cultures (J. Cell Biology, 32: 297-308, 1967). Gevaudan, Ray, and Arnaud reported short-term growth of primary rabbit and continuous human cell lines in peptone supplemented 199 (Annales de l'Institut Pasteur, 100: 821-824, 1961). The objective of this study was to evaluate these supplements, alone and in combinations, for initiation of and improved growth of primary cell cultures.

Methods Employed:

Twelve- to 14-day C3H/HeN mouse embryo cultures were initiated in the usual way. The growth medium consisted of NCTC 135 supplemented with either Bacto-peptone (10% v/v), Peptic peptone (10% v/v), Witte's peptone (15% v/v), albumin (40 mg/ml), or α_1-globulin (2 µg/ml)(Grand Island Biological Co). Culture fluid was replaced with fresh medium as required and the cultures were subcultured (passaged) when the cell population formed a confluent sheet.

Major Findings:

Cultures initiated in NCTC 135 supplemented with Bacto-peptone or Peptic peptone usually died within 3-6 weeks, suggesting these peptones are unsuitable for primary cell culture. Those initiated in Witte's peptone supplemented medium NCTC 135 grew slowly, but have survived through several culture transfers. Relevant information is presented in Table 1:

Table 1

Experiment No.	Date Initiated	Number of Culture Passages	Supplements added to NCTC 135
7318	5 Aug. 71	8	Witte's peptone
7612	18 Jan. 72	5	Witte's peptone
7613	18 Jan. 72	4, 5, 6	Witte's peptone
7416	21 Sept. 71	11,12,13	Witte's peptone Albumin
7353	24 Aug. 71	8, 9	Witte's peptone Albumin α_1-globulin

Significance and Proposed Course:

Though still in their preliminary stages, these studies represent an attempt to establish cells in culture in the absence of whole serum. Since the malignant conversion of these cells is influenced by the type of serum used, peptones may offer a supplement which either potentiates the growth of neoplastic cells in vitro (which presumably are not present in low passage, embryonic tissue), or retards the rapid proliferation--and hence the dedifferentiation process--seen in low passage level, serum-supplemented cell cultures. The latter may explain the slow growth observed to date in cultures initiated in Witte's supplemented NCTC 135. To date, both albumin and α_1-globulin have failed to support the degree of growth suggested by previous reports (see Objective for references).

Future work includes: (a) additional attempts to initiate fresh tissue (primary cell cultures) in NCTC 135 supplemented with 15% Witte's peptone, Witte's peptone plus albumin and α_1-globulin, 10% Peptic peptone, 10% Peptic peptone plus albumin, and Dulbecco-Vogt's modified MEM supplemented with 15% Witte's peptone; (b) Cytologic and chromosomal analysis of all cell strains mentioned in Table 4 (so as to predict the malignant conversion) or(a) above; (c) injection of these cell strains into irradiated, syngeneic mice to determine if the cells have undergone malignant conversion in vitro; and (d) preliminary purification of Witte's and Peptic peptone by dialysis. Both the dialyzable and nondialyzable fractions will be tested for their growth promoting activity for primary cultures and established cell lines (RH, L-M). Preliminary studies suggest that, like Bacto-peptone, the activity of Witte's peptone is dialyzable.

4. Preliminary biochemical and biological analyses of whole Bacto-peptone dialysate.

Objective: Pumper et al. (Nature 207: 662-663, 1965) demonstrated that the biological efficacy of whole Bacto-peptone could be purified 25-fold by "reverse dialysis" without any loss in biological activity. We have shown (Proc. Soc. Exptl. Biol. & Med. 139: 96-99, 1972) that the dialyzable components (PD) could be separated into two biologically active fractions by Sephadex G-10 chromatography. This present study is an expansion of the above; the emphasis has been placed upon identifying the chemical character of the biologically efficacious components in PD with the ultimate aim of either designing a new, simple culture medium, or incorporating the efficacious compound(s) into currently available media.

Methods Employed:

A. Media and Cell Cultures:

Powdered medium 199 was prepared as a 2x concentrate in triple glass distilled water. (The final glutamine and sodium bicarbonate concentrations were 100 mg/liter (2.0 mM) and 1250 mg/liter, respectively). The fluid was Millipore filter sterilized and diluted with an equal volume of sterile, triple glass distilled water and supplement as required; Bacto-peptone dialysate (PD) was used as a final concentration of 2.5 mg PD/ml culture medium. All

cultures, stock and experimental, were gassed with 10% CO_2-air. No antibiotics were used; all analyses for PPLO were negative.

Variations in the culture media used in each experimental bioassay was minimized by preparing a single pool of unsupplemented base medium. Equal volumes of this pool were distributed to sterile Florence flasks. The experimental variables were reconstituted in triple distilled water, sterilized, and added to a volume (usually 5 ml) sufficient to yield 1x medium. The osmolality of each experimental medium was determined with an Advanced osmometer (Advanced Instruments, Inc., Newton Highlands, Mass.)

Pumper's rabbit heart cell line and a derivative of Merchant's L-M line were subcultured continuously in medium 199 plus 2.5 mg PD/ml (199-PD). Parent cultures were transferred weekly with 2 intervening fluid renewals. For nutritional studies several parent T-15 culture flasks were seeded with approximately 10^6 cells. After 72 hours incubation at 37.5° ± 0.15°, the culture medium was removed and fresh growth medium added to each flask. Twenty-four hours later, the growth medium was replaced with unsupplemented medium 199 and the culture incubated for an additional 18-24 hours; at this time the cell sheets were dispersed with collophane mops, the cells resuspended in unsupplemented medium 199 at density of about $4\text{-}5x10^4$ cells/ml, and replicate cultures prepared as previously described (see section 1). After 2-3 hours incubation, at which time greater than 99% of the cells had attached, sets of 5-6 randomly selected replicate flasks were prepared, and the planting medium replaced with medium 199 supplemented with the appropriate experimental variable. To evaluate marginal biological responses, replicate control cultures were incubated in 199 supplemented with 0.0, 0.5, and 2.5 mg PD/ml culture medium. After 48 hours incubation, the growth medium from each experimental set was replaced with fresh medium, and after an additional 72 hours incubation the cell population in each flask was harvested and counted on a Coulter Model B counter.

B. Biological Analyses:

To evaluate the trace metals, ether soluble material, and acid digestible component(s) in PD, an amount of PD sufficient to supplement an equal volume of growth medium at 2.5 mg PD/ml culture medium was treated, as described below.

i) Trace metals.--Dried PD was ashed at 610° ± 10°F for 24 hours. The residue was converted to the sulfate form with a small volume of 36 N H_2SO_4, and the liquid subsequently evaporated by heat. The sulfates were dissolved in triple distilled water and the solution autoclave sterilized prior to addition to the base medium. The "acid control" was prepared identically by adding a small volume of 36 N H_2SO_4 to a clean crucible.

ii) Ether soluble material.--Dried PD was reconstituted in a small volume of triple distilled water and the solution extracted once with reagent grade diethyl ether (5 ether:1 H_2O, v/v). Typically, 50 ml of ether were used per 100 mg PD. The phases were collected, evaporated to dryness, redissolved, and sterilized in triple distilled water prior to use. Reconstitution was effected by mixing equal volumes of the redissolved, sterile material. No water insoluble material was apparent in the organic phase.

iii) Acid hydrolysis.--Dried PD was reconstituted and autoclaved in a volume of triple distilled water sufficient to contain all the PD for any variable in 2.0 ml. This volume was added to each of several acid cleaned digestion tubes (Virtis). Redistilled HCl (sequanal grade, Pierce Chemical Co., Rockford, Ill.) was diluted to twice the normality desired, and 2.0 ml of acid aseptically added to the PD. The fluid was immediately frozen, evacuated ($\sim$.5 mm Hg), and sealed. Hydrolysis was carried out at either 37.5 ± 15° or 104° ± 2° for 22 hours. Following hydrolysis, the samples were neutralized either by the addition of an equal milliequivalence of NH_4OH or by flash evaporating the acid from the sample. The samples were lyophylized, then reconstituted in 5 ml triple distilled water and autoclaved.

iv) Gross vitamin assay.--To evaluate if the biological response to PD might be due to the presence of an unknown or to an additional critical amount of some vitamin, MEM vitamins (Grand Island Biological Co., Grand Island, N.Y.) were superimposed on those vitamins present in medium 199 as a 1x or 2x supplement, both with and without whole PD. In addition, replicate cultures were grown in NCTC 135 or Dulbecco-Vogt's modified MEM; both these media were prepared in this laboratory, and respectively represent a source of several vitamins not found in MEM or 199 and a source of a higher concentration of certain vitamins.

C. Chemical Analyses:

i) Trace metals.--The arsenic, lead, and chloride content of PD were quantitatively determined by atomic absorption spectrophotography. Analysis for the other inorganic elements reported was by emission spectrometry.

ii) Carbohydrate, nucleoside, and nucleotide analysis.--The presence of carbohydrate in untreated PD was evaluated by the Molisch test. Nucleoside and nucleotide analyses were performed by hydrolyzing PD in 0.1 N HCl for 40 min. at 90°; a Molisch test was performed on the hydrolysate.

iii) Ultraviolet spectrum.--The UV spectrum of whole PD was obtained with a Perkin-Elmer recording spectrophotometer using a 1.0 mg PD/ml sample.

iv) Amino acid analyses.--For total amino acid content, dried PD was hydrolyzed in 5.8 N HCl (sequanal grade, Pierce Chemical Co.) for 22 hours at 104° ± 2°C. Samples were analyzed by the method of Spackman, Stein, and Moore (Anal. Chem., 30: 1190-1206, 1958). For "bound" and "free" amino acid determinations, dried untreated PD was resuspended in pH 2.25 citrate buffer and analyzed as a physiological fluid. All analyses were performed on a Beckman 120 C automated amino acid analyzer.

Major Findings:

i) Trace metal analyses.--Studies suggested that the inorganic metals in PD slightly enhanced cell growth, particularly when little or no whole PD was added to the growth medium (i.e., at 0.0 and 0.5 mg PD/ml culture medium wherein the final cell population is reduced). Quantitative experiments were performed both with RH-PD and L-M (PD) cells; in a typical experiment, cell populations 1.5-2 times greater than the control were observed when 1x ashed PD was incorporated into the growth medium.

Data obtained by quantitative inorganic analysis of whole Bacto-peptone, PD, and the nondialyzable fraction of whole Bacto-peptone showed an apparently higher concentration of zinc in the biologically efficacious PD; this element is particularly significant in in vitro cellular metabolism (Cox, R.P., Mol. Pharmacol. 4: 510-521, 1968).

ii) Growth following ether extraction of PD.--Aqueous PD was extracted with diethyl ether to evaluate the presence of lipid soluble components, such as the fat soluble vitamins. The material separating in the aqueous or organic phases was used as a supplement for RH (PD) and L-M (PD) cells. A predominant amount of starting material was recovered in the aqueous phase, and when used as a sole in vitro supplement this material supported cell growth equivalent to that obtained with untreated PD at 2.5 mg PD/ml. The ether soluble material represented less than 1% (w/w) of the starting material, and at this concentration possessed no apparent biological efficacy.

Since many vitamins are required only in trace amounts, extraction of such entities from the PD might go unnoticed if a sufficient concentration remained in the intracellular pool of the experimental cultures. Because the RH (PD) cells fail to grow in PD-free medium, these cells were continuously subcultured in medium 199 supplemented with the "water phase components" of PD at 2.5 mg water phase material per ml culture medium. No deficiency syndrome nor lag phase were observed through 17 transfers (3-1/2 mo.) in medium 199 supplemented with the water phase material, as compared with stock cultures grown in 199-PD. Hence the activity is fully soluble in water, and isn't due to the presence of a lipid soluble vitamin.

iii) Gross vitamin assay.--To determine if a mixture of vitamins could support growth and survival of cells adapted to growth in 199-PD, Eagle's MEM vitamin mixture was superimposed on medium 199. Either 1x or 2x vitamins were used with different concentrations of PD. Both cell lines tested showed a marked cell population increase in replicate cultures incubated with the 1x MEM vitamin supplemented, PD-free 199, and a marginal increase in cultures incubated in medium 199 supplemented with 1xMEM vitamins and 0.5 mg PD/ml. This demonstrates that both cell lines will grow to a higher population density with an increased vitamin supplement.

iv) Acid digests of PD.--To determine if the biological efficacy of PD could be destroyed by acid, aqueous preparations of PD were hydrolyzed as described, the acid neutralized or removed, and the residue added to culture medium. In general, PD subjected to low acid concentrations (0.003-0.09 N HCl) promoted a greater degree of growth than untreated PD. At acid concentrations equal to and greater than 0.15 N, the cell population observed after 5 days incubation was markedly lower. On the surface these results support the theory that the efficacious component(s) of PD is peptide in nature, and can be explained as rendering the peptide and/or constituent amino acids more "available" for utilization, until a point at which the sample was digested beyond nutritional usefulness. The methods of neutralizing the acid, however, are inadequate, and the lack of cell growth observed at higher acid concentrations probably is due to additional salts introduced in the neutralization process (making the medium hypertonic). Hence, the data collected to date can only be used to support similar studies which are either planned or in progress.

v) Carbohydrate, nucleoside, and nucleotide analysis.--These analyses were performed to detect carbohydrate at a concentration of no more than 1%. All tests were frankly negative, suggesting that if any carbohydrate, nucleoside, or nucleotide (such as cyclic AMP) were present, it was present at less than 1% on a weight for weight basis.

vi) UV absorption pattern.--A UV absorption spectrum of PD indicates a λmax in the 230 µm range, with a flat broad lower peak in the 250-280 µm region. This is in contrast to published reports of the λmax of similar preparations being in the 260-265 µm region (Amborski, R.L., and Moskowitz, M., Exptl, Cell Res. 53: 117, 1968; Yamane, I. and Murokami, O., BBRC 45: 143-146, 1971). The preparation of PD differs from the method used by Amborski-Moskowitz, and therefore, the materials may not be strictly comparable. The substance reported by Yamane and Murokami was recently reported in a preliminary note and could be present in PD. However, the full details of its isolation are not yet available.

vii) Amino acid analyses.--Owing to the fact that whole Bacto-peptone is an enzymatic digest of animal tissue, and that PD gave a mixture of ninhydin and hypochlorite reactive spots in both descending paper chromatography and high voltage electrophoretic systems (Pumper, R.W., and Taylor, W.G., unpublished data), it has been assumed that the biological efficacy of PD was due to a pharmacologically active, hormone-like peptide(s). That whole PD is carbohydrate, nucleoside and nucleotide, and lipid negative (within the limits of the tests used) indirectly lends support to this thesis, as does the UV absorption pattern (a peptide band absorbs maximally in the 230 µm region). Amino acid analyses were performed on hydrolyzed and unhydrolyzed PD samples; the results demonstrate the presence of high percentage of "bound" amino acids--particularly aspartic acid, proline, glycine, and methionine--presumably in the form of a small dialyzable peptide. Demonstrating that these peptide(s) are the cause of the biological efficacy remains to be completed; the enhanced growth observed with partically acid hydrolyzed PD supports this conclusion.

viii) The role of glycine peptides.--Based upon earlier amino acid analyses which demonstrated a high total glycine content, a variety of glycine peptides were incorporated into NCTC 135. These studies indicated that glycine peptides exhibited a slightly stimulatory effect on strain L (grown in fully defined NCTC 135) (Taylor, W.G., unpublished data). A pilot study with RH (PD) cells suggested the same response, but RH cells could not be subcultured in glycine-peptide supplemented medium which was devoid of PD. These results, however, are preliminary, and quantitative studies need to be performed.

Significance and Proposed Course of Study:

The further improvements of currently available tissue culture media--with particular emphasis upon the development of a milieu which promotes the stability of cultured mammalian cells, both normal and neoplastic--requires a new evaluation of old problems. A better understanding of in vitro cellular requirements and a reevaluation of the synthetic media, the gaseous environment, the osmolality, viscosity, and redox potential of the fluid, and the use

of undefined biologics such as serum are all essential. PD represents a supplement as potent for peptone adapted cells as serum for most known cell lines. For example, if 1.5×10^5 RH (PD) cells are planted in a T-15 flask, a typical cell population after five days incubation approximates 45×10^5 (30 fold increase). Though definitive proof is lacking, these cells are probably malignantly converted--like HeLa and Strain L-929, for whose nutritional needs most of the currently available culture media have been formulated. Thus, this cell line (because it has been grown for several years in the absence of serum) may provide important clues towards the development of better, defined culture media, particularly for neoplastic cells including human tumor cells.

Ongoing and future studies include the following: a) analytical (microbial) vitamin assays on PD, and the testing of synthetic vitamin mixtures prepared from these data; b) enzymatic digest of PD, in an attempt to destroy activity by destroying peptide bonds; c) testing of synthetic amino acid mixtures prepared from the analyses performed to date; d) the use of ultrafiltration, ion exchange, and gel filtration chromatography, and other appropriate techniques to further purify PD, with the appropriate biological assays.

5. Evaluation of some organic buffers for cells in vitro.

Objective: Since the introduction of zwitterion organic buffers by Good (Biochemistry 5: 467, 1966) a limited number of studies have been undertaken to evaluate their usefulness for stabilizing the pH of tissue culture systems. The purpose of this study is: (i) to evaluate the usefulness of such buffers in chemically defined medium (CDM), and simultaneously study the physiological requirement for bicarbonate by cells in vitro, and (ii) to evaluate the role, if any, of bicarbonate in in vitro carcinogenesis.

Methods Employed: HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) was prepared as an aqueous 0.25 M solution (10x). This was used to buffer chemically defined medium (CDM) NCTC 135 with: a) varying HEPES concentrations with a constant bicarbonate level, and b) varying sodium bicarbonate levels with constant HEPES concentration. The use of oxaloacetic acid and sodium fumarate as in vitro supplements was studied also.

Major Findings: Preliminary studies with strain L (NCTC 2071) grown in fully defined medium NCTC 135 suggested that strain 2071: (i) could proliferate and survive in medium containing reduced amounts of sodium bicarbonate, (ii) was not adversely affected when HEPES was added to CDM, and (iii) required more bicarbonium ion than is produced by the catabolism of glucose, as evidenced by the cell maintenance in medium prepared in PBS. Subsequent studies have shown that, in the presence of increasing concentrations of HEPES buffer (0-50 mM), the best growth was observed as buffer concentrations of 10-20 mM HEPES. When 10 mM HEPES was employed with varying sodium bicarbonate concentrations, a pH shift was observed with culture medium containing greater than 800 mg sodium bicarbonate/liter. Fair growth was observed in cultures incubated in NCTC 135 containing 800 mg or less sodium bicarbonate/liter, and preliminary studies suggested that 1-2 mM oxaloacetic acid enhanced growth, even in the absence of sodium bicarbonate. This study, however, has been disappointing considering the large number of experiments which have failed

partly or completely--in some cases for reasons unknown. It is possible, despite the claims of Eagle and coworkers (PNAS, 68: 229-233, Nature New Biology 233: 271-273, Science 174: 500-503) that these buffers simply are not suitable for cells grown in fully or partially chemically defined growth medium, perhaps due to a "toxicity" not observed in serum-supplemented growth medium.

Significance and Proposed Course:

The importance of maintaining a stable pH is obvious, particularly in a biological system. The inability to use successfully Good's organic buffers has been reported by several laboratories, but their potential benefits dictate that further studies be carried out. At the present, various modifications in the growth medium are under consideration and once completed attempts will be made to initiate cells in bicarbonate-free medium to evaluate their spontaneous neoplastic conversion. Until this is accomplished, a variety of continuous lines with high glycolytic rates will be used to test and improve the experimental media developed.

Publications:

1. Rutzky, L.P., Taylor, W.G., Pumper, R.W.: In vitro stimulation of rat liver cells by homologous partial hepatectomy serum. In Vitro 7: 1-7, 1971.

2. Taylor, W.G., Taylor, M. L., Lewis, N.J., Pumper, R.W.: Studies on a serum substitute for mammalian cells in culture. I. Biological efficacy of whole and fractionated peptone dialysate. Proc. Soc. Exp. Biol. Med. 139: 96-99, 1972.

3. Taylor, W.G., Price, R.M., Dworkin, R.A., Evans, V.J.: A simple device for planting replicate mammalian cell cultures. In Vitro 7: 00-00, 1972. In press.

Serial No. NCI-3262 NONE
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The development of a homeostatic cell culture system and/or growth promoting system for cells in vitro.

Principal Investigators: W. G. Taylor and V. J. Evans

Other Investigators: K. K. Sanford and R. R. Gantt

Cooperating Units: V. P. Perry, C. Kroner, American Foundation for Biological Research, Biological Research Division, Rockville, Maryland

Project Description: Development and testing of a pulsatile nutrient fluid perfusion system to grow mammalian cells in vitro under homeostatic control and/or to develop maximum population potential of mammalian cells in vitro. Objective 2. Approach 4 (specifically 2.4.3.8 or 2.4.3.3)

Objective:

Current cell culture media and particularly techniques subject the cell to a myriad of physical and chemical variations. The generally used system of twice or thrice weekly culture media renewals leads to fluctuations in pH, radical temperature change, and ultimate depletion and perhaps even exhaustion of critical factors such as glucose and amino acids. This constant physiochemical variation undoubtedly influences the growth of the cells. At the least, log growth is interfered with so that maximum population potential is not achieved. Also, these fluctuations may have bearing on the spontaneous malignant transformation that occurs in cells explanted in vitro.

Our objective is to develop homeostatic culture techniques through the use of a pulsatile perfusion system. Planned is the ability to monitor and automatically control pH, pO_2, pCO_2, glucose utilization, and amino acid utilization; biological markers include the influence of physiologic (including nutritional and biophysical) variation on cell population changes, morphology, chromosomal stability, and ultimately neoplastic (malignant) conversion.

Methods Employed and Results:

A continuous pulsatile perfusion system has been designed to feed continuously cells in vitro. To date the system has been tested (by the purchase order mechanism) on a human diploid skin strain. Cells have been grown for 36 days with a several-fold increase in population on recirculated fluid, and finally, maintenance of a stable but viable population. Photomicrographs of population changes are available. The system, therefore, appears highly feasible for the growth of cells.

The system is now being altered to incorporate monitoring devices for pH, pCO_2, pO_2, and glucose utilization. Further, the system is being modified to add all new media or depleted components to study their influence on cell growth. Basically, the system operates in the same way as the mammalian cardio-vascular system, and employs the Carrel-Lindberg-Rockefeller pump principle.

Proposed Course of Work:

It is planned to align a number of cultures in series with the monitoring instruments and culture medium supplement reservoirs. The effects on growth of human diploid cells and of mouse cells will then be determined particularly with respect to neoplastic (malignant) conversion of the latter and also to study the effects on growth of mass amounts of mammalian cells under automated conditions especially human cells for biochemical studies, virus or interferon production, and biologically active compounds produced by cells.

Significance:

The major significance is to provide a system with minimum trauma (physiologic, biologic, and biophysical) to the cells in culture. Therein we can monitor, control, and study a number of factors that could influence the malignant conversion of cells in vitro and thus, hopefully, obtain by control of these factors a normal or at least non-malignant cell population. In addition, there should be a number of ancillary benefits from the use of such a pulsatile perfusion system. We anticipate that mass amounts of cells might be grown in this system -- such cells as lymphocytoblasts, cells producing biologically active components not yet synthesized in the laboratory at all or only in minute amounts, namely certain glycoproteins, hormone-like substances, etc. Virus producing cells also could be grown in large amounts for vaccine production in such a system. The system is so designed that it is unusually non-traumatic to cells and might well be used for growth of interferon producing cells in the absence of serum or at the very least small molecule protein additives. Present methods of producing interferon usually require serum-supplemented tissue culture medium (thereby introducing a large quantity of antigenic material into the fluid), but even so, the quantities of interferon produced are impractical for commercial therapeutic products. Presumably a greater number of cells grown under ideal metabolic conditions would produce greater quantities of this antiviral agent. Recalling that mouse liver epithelial cells from early studies in this laboratory where cells were subjected to daily fluid renewal produced as many as 30×10^6 cells/ml of nutrient fluid in routine suspension cultures, we might well expect yields in this system the equal or higher without the need to resort to expensive hand labor. We visualize the system as ultimately being completely amenable to automation.

The single Objective, Approach, Approach Element, and Project Area this project most closely supports is probably 2.4.3.8, but it is certainly applicable in 3.2, and indirectly in 5.5, 6.1, 6.2, 6.3, and 6.6 as its success will supply a need in many areas.

Laboratory of Cell Biology

SUMMARY

July 1, 1971 - June 30, 1972

Lloyd W. Law

Some highlights of achievements in the Laboratory of Cell Biology

Studies of the biologic properties of solubilized, purified transplantation antigens (H-2) and of virus-specific tumor antigens have continued with the following pertinent findings:

Using an F2 fraction of s lubilized H-2 antigen (specified by the H-2^a gene) and containing the major H-2^a specificities, it was possible to show that this material has enhancing properties and also induces immunologic tolerance in appropriate hosts. Soluble antigen-treated CBA and B.10D_2 mice grew the allogeneic A strain tumors Sarcoma 1 and YAA-C1; controls did not nor did those mice receiving the F3 fraction known to contain predominantly non-H2 gene products. Passive transfer of enhancement was also achieved and the response was shown to be specific - that is anti-H-2^a enhancing antisera did not enhance non-H-2^a allogeneic tumors.

The biologic completeness of the F2 fraction was also further tested in immunologic tolerance studies. B.10D_2 strain mice were treated from birth, daily, with the F2 fraction and then assayed for their cell-mediated and humoral antibody (cytotoxic and hemagglutinins) responses. The antigen-treated mice retained their capacity to reject B.10A congenic skin grafts but were found to be tolerant to humoral antibody induction. The majority of mice were unresponders and tolerance was found to persist for approximately 150 days after termination of antigen treatment. Immune tolerance was found to be specific in that the treated B.10D_2 mice responded normally to H-2^b antigens (not shared by H-2^a). Preliminary evidence also indicates a striking reduction in enhancing antibodies - as well as reduction of cytotoxins and hemagglutinins.

Attempts are continuing to further characterize the membrane-bound tumor antigens, virus specified VSTA, with the objective in mind of solubilizing and purifying these. Two systems have received attention: mKSA tumor of (BALB/c) mice adapted to in vivo and in vitro assays; this neoplasm contains strong SV40-specified transplantation antigens. RBL-5, a lymphoid neoplasm, has been shown to be strongly immunogenic and contains tumor antigens of the transplantation type specified by Rauscher leukemogenic virus.

Crude soluble fractions of mKSA tumor cells inhibit specifically the cytotoxicity of anti-mKSA antisera; to date however this fraction has not been shown to be immunogenic in vivo; nevertheless the crude membrane fraction is immunogenic which provides hope that mKSA antigen can be solubilized and purified under proper conditions.

Crude soluble (CS) and, a G-150 Sephadex fraction, F2, similar in composition to the H-2^a F2 fraction, described above, have been shown to provide resistance to the transplantation of RBL-5 leukemic cells. In vitro monitoring of the purification steps are now possible in this system since anti-RBL-5 sera are cytotoxic and the crude soluble and F2 fractions of antigen inhibit the cytotoxicity. No infective leukemogenic virus has been found in the CS or F2 fraction, strengthening previous observations recorded from this laboratory that these tumor antigens, induced by the RNA viruses of the leukemia-sarcoma complex, are "new cellular" antigens and not structural viral antigens.

A microcytotoxicity assay (MCA) modified from the colony inhibition test has been utilized in two separate studies:

In the first, the MCA was compared with another in vitro measure of cell-mediated immunity, a 51-chromium-release assay (CRA). The two methods were employed concurrently to assess the cellular immune response to a transplanted, syngeneic, Gross virus-induced lymphoma in W/Fu rats. These studies indicated that the information provided by these two assays, tested under a variety of experimental circumstances was essentially equivalent, and that the activities measured by both methods parallel the existing state of tumor immunity in the intact host, as revealed by standard transplantation tests.

In the second, the MCA was used to determine the cell-mediated immune response in mice acutely infected with lymphocytic choriomeningitis (LCM) virus; lymphoic cells from mice receiving LCM virus were shown to specifically inhibit the growth of LCM-infected fibroblasts in vitro. The cytotoxic activity of lymphoid cells from mice immunosuppressed by various means was depressed. These studies indicate that a lymphocyte-dependent, serum complement-independent immune mechanism is of primary importance in the mediation of acute LCM virus-induced disease. These studies have also demonstrated that the MCA can also be utilized to detect marine leukemia virus antigens expressed by non-transformed, virus-infected fibroblasts.

Studies of Glycolipids. a) Changes in DNA virus transformed established cell lines. In Swiss mouse cell lines after transformation with SV40 or polyoma virus, there was shown to be a reduction in the activity of an enzyme (uridine diphosphate N-acetylgalactosamine: hematoside N-acetylgalactosaminyl-transferase) necessary for the synthesis of the higher gangliosides. These changes were found to be reversibly coupled to the changes in growth properties in tissue culture: in the flat derivatives of the virus transformed cell lines which were selected out by an FUDR treatment as revertants to normal phenotypic growth properties in culture, there was a trend of reversion in ganglioside pattern and in enzymatic activity near to that of the parent 3T3 line, in spite of the continued presence of the viral DNA.

Mouse cells productively infected with polyoma virus did not show decrease in the aminosugar transferase activity. Cocultivation of virally transformed cells with enzymatically active cells did not result in reduced enzyme activity in the enzymatically active cells, or in the restoration of enzyme activity in the virally transformed cells. Similarly, no change in enzyme activity occurred when admixing homogenates of the two type of cells.

Similar results were obtained in BALB/c cell lines transformed by SV40 and in certain "flat" derivative cell lines selected out by H. Smith and C. Scher by a differential need for serum growth factor requirement. The "flat" cells continue to carry the viral genome but are subject to contact inhibition of growth in culture and show also the reversal in the transferase activity and in higher ganglioside content. The enzyme depression does not occur in lytic, virus-producing infection, when the whole virus genome is being transcribed and translated. Thus the enzyme depression appears to pertain specifically to the viral function(s) involved in cell transformation. But insertion of the virus genome into the cell genome (and continued expression of the T antigen) is not sufficient: the "flat revertant" cells can be looked upon as variant cells which are capable to repress stably the growth property alteration by the inserted virus genome. Significantly, these cells also depress the depression of the transferase. Thus the activity of the transferase is controlled by cells conjointly with the functioning of that part of the virus genome which transformed growth property in culture.

b) RNA virus transformed cells. The Swiss 3T3 cell line was carefully titrated with a Moloney sarcoma virus (MSV) to find the conditions of transformation which results in essentially 100% of cells being morphologically transformed in about 48 hours. There were no changes observed 48 hours after transformation, however, after 96 hours the N-acetylgalactosaminyl transferase activity decreased to 50%, and after seven days to 20% of the original activity. This type of transformation kinetic experiments avoid the dual problem of selection and of effect of long range continuous cultivation (see below). Previous less controlled work in established cell lines (BALB/c, Swiss, AKR) and in their MSV or KiSV (Kirstein sarcoma virus) transformed derivative lines, revealed no reproducible differences.

Function of viral genome: In SV40 experiments, "cryptic transformants" revealed some very intriguing nature of the malignant tranformation of cells. They have the complete copy or copies of viral genome integrated into the cells, but most of their functions were repressed: they can synthesize T antigen, they can produce tumors in vivo but show contact inhibition in vitro. When tested for TSTA or TSSA, neither was detected. It indicates that the genes which code for these antigens are not expressed.

A study was made of fetal antigens in tumor cells and their relationship to tumor specific antigens of papova virus-induced tumors. Sera which reacted specifically with the TSSA or polyoma (APS) or SV40 tumors (ASVS) did

not react with fetal tissue cells and the activities of these sera could not be removed by the absorption with large quantities of fetal tissue. This indicates that fetal tissues do not have detectable amounts of antigens which will cross-react with the TSSA of papova virus-induced tumors. In contrast, the antisera produced against fetal tissues will react with various tumor cells in addition to fetal tissue cells, and the activities of these sera against polyoma or SV40 tumors could be removed by absorption with various nonpolyoma or nonSV40 tumors. Therefore, our findings indicate that fetal antigens are expressed in cells after malignant transformation, but they are different from tumor specific antigens which are specific for a particular tumor or tumors induced by a particular virus.

Studies of tumor associated antigens in breast carcinoma. By using the cell line, F-230, derived from a patient with breast carcinoma (Old et al.) the reactions obtained with the sera from patients with breast carcinoma seemed to be more related to HL-A antigens than to truly tumor specific or tumor associated antigens. It may be due to the absence of specific antibodies in these sera, or the F-230 cells do not have the tumor specific or tumor associated antigens.

A new assay for cell-mediated cytotoxicity for monolayer tissue culture cells has been developed in our laboratory. Cytotoxicity is measured by the release of ^{125}IUdR from labeled target cells. This has proven useful in the study of the immune response to Gross leukemia in rats and to human tumor associated antigens.

The relationship of various assays for cellular immunity with each other and with the in vivo resistance to tumor have not been clearly defined. In the Gross leukemia system a study has been performed to compare the results of tests with the short-term ^{51}Cr-release cytotoxicity method with the results of the Takasugi-Klein microcytotoxicity method (as modified by the Hellstroms). These two assays seem to be measuring similar events; many of the differences observed could be accounted for by the in vitro activation phenomenon noted above. Studies are now in progress to compare these two assays with the IUdR cytotoxicity method.

An inhibition technique has been developed in our laboratory for studying the specificity of the cellular cytotoxic reactions. This technique has already provided information on the specificity of the reaction against Gross leukemias in ratw and against MSV in mice. The specificity of the cellular reactions are different from the specificities of antibodies. Antigens have been detected on cells transformed byMSV, which do not produce any infectious virus. This provides strong evidence that this specific cell surface antigen is distinct from virus envelope antigen.

The sera of most mouse strains contain natural antibodies to antigens on plasma cell tumors in BALB/c mice. One of the antigens detected in these sera has been shown to be identical to the PC-1 antigen described by workers at Sloan Kettering. The sera also contains antibodies to a viral envelope antigen (VEA) of viruses found in plasma cell tumors. This VEA is distinct from that associated with Gross virus. Evidence suggests that the observed

immunological reactions may be related to a new C-type virus, associated with plasma cell tumors.

Patients with neoplastic diseases have been shown to have delayed hypersensitivity reactions to extracts of tumor cells. Delayed skin reactivity in patients with acute leukemia has been found to correlate with a remission status of the patients. Several patients have become skin test negative when they relapsed and tests became positive again when they went back into remission. Allogeneic skin tests have given some positive reactions, indicating the presence of some common antigens in acute leukemia. Studies have been performed on patients with carcinoma of the colon and breast and with melanoma. Colon tumor extracts contain a carcinoembryonic type of antigen which elicits skin reactions; this antigen has been shown to be distinct from the carcinoembryonic antigen of Gold. In melanoma, soluble antigens of two different sizes have been identified. Reactivity to the higher molecular weight antigen appears to correlate with stage of disease.

The lymphocytes of almost all normal individuals have been found to have cytotoxic activity against human lymphoid cell lines in the ^{51}Cr release assay. Patients with cancer or immunosuppression have decreased or no activity. Studies are in progress to determine the nature of this reactivity.

We have attempted to confirm the observation of the existence of plasma cell tumor antigens and to identify the nature of the tumor antigen in particular to determine if it is related to immunoglobulin idiotypes or to the PC1 antigen. It has been found that mice bearing AdjPC5 can be cured as reported and that mice which have regressed large grafts can resist challenge with AdjPC5 cells. Mice immunized with other tumors have not yet produced immunity to AdjPC5, suggesting that there are several types of antigens.

Thus far all experiments have been carried out with subcutaneous grafts. Experiments are planned to test intraperitoneal graft and to then determine if immunized mice which have previously received intraperitoneal injections of mineral oil can reject the challenges.

In a continuing investigation of intracisternal A-particles, virus-like particles which are found in a variety of normal and neoplastic mouse tissues, certain distinctive characteristics have been established. Particles isolated from several different mouse myeloma lines and from cultured neuroblastoma (C1300) cells all contain a major structural protein with an apparent molecular weight of 70,000 as estimated by electrophoretic mobility in SDS-containing polyacrylamide gels. The size distribution of A particle proteins is different from that observed for extracellular murine leukemia and mammary tumor viruses. Rabbit antiserum has been developed that reacts with the major structural protein in both C-F and immunodiffusion assays. The antigen can be detected in A-particle isolates from tumors, but not in preparations from normal mouse organs or samples of leukemia and mammary tumor virus. Conversely, isolated A-particles do not react in C-F or immunodiffusion assays with antisera against leukemia virus antigens. Recently

an extract from 30 grams of BALB/c mouse embryo tissues was shown to contain antigen and particles as seen by electron microscopy. This finding plus the fact that A-particles are present in both male and female gonadal tissue make it most probable that certical transmission takes place, a feature shared in common with the leukemia viruses. Experiments have been initiated which will yield data concerning the presence of: (1) a reverse transcriptase; (2) the presence of a high molecular weight RNA of the type seen in the known oncogenic RNA viruses; (3) the rate of synthesis and degradation of particles in both animal and tissue culture systems. It is anticipated that these results will help determine whether or not A-particles reflect the expression of a viral genome as opposed to the abnormal production of a cellular constituent.

The mouse has been a rich source of homogeneous immunoglobulins with antigen binding activity. Some 16 proteins are under study in this laboratory that have binding activity for a variety of different polysaccharide moieties, phosphoryl choline and nitrophenyl groups. For many of these the specific chemistry of the ligand is known but for others only an antigen has been identified and it becomes essential to fine the chemical nature of the determinant group. We are preparing various mono- and oligosaccharides from antigens and testing them as inhibitors in specific systems. Work has been completed on identifying highly active inhibitors for two new myeloma proteins SAPC-10 and TEPC-191; the active inhibitors are oligosaccharides containing 2, 3 or 4 beta 1-6 linked D-galactose. We are currently attempting to establish the binding constant for the di- and trisaccharides. Further insoluble immunoabsorbants have been prepared which makes it possible to purify these proteins in a single step.

Protective immunity can be established in BALB/c mice such that they are resistant to induction of tumors by a small number of malignant plasma cells. We have begun experiments aimed at investigating the role of the secreted immunoglobulin in this immunity. So far mice repeatedly immunized with a two-chain IgA protein have not shown any protection against challenge with the tumor cells (MOPC 88) producing this protein. Another approach being tried is to see if an xenogeneic antiserum to a secreted light chain can transfer any protection to a challenge of tumor cells from the tumor that secreted the light chain. If conditions can be worked out for some success with this antiserum, experiments in human patients will be considered. To this end certain human K chains are being purified and used to induce a precipitating or cytotoxic antiserum in horses and goats.

We have succeeded in crystallizing the combining site of two myeloma proteins with activity to phosphorylcholine, McPC 603 and MOPC 167. The McPC 603 crystals give a high degree of resolution (2.7°A) in preliminary X-ray defraction studies. Studies are in progress to determine both the primary and tertiary structure of this molecule which should produce the first three dimensional model of an antibody combining site. Attempts to crystallize the combining sites of other myeloma proteins and to determine their primary structure are in progress.

It is felt that studies of this nature will lead to the eventual in vitro design and synthesis of antibody molecules with determined specificities. These molecules would be of an extreme diagnostic as well as possibly therapeutic value.

Recent reports using sensitive radio-immunoassay methods have shown that αFP Alpha-Fetoprotein no longer is associated exclusively with hepatocellular carcinoma and embryonal cell carcinoma, but is elevated in a wide variety of neoplasms and liver disease and is actually present in normal adult serum in ngm/ml amounts.

There has been set up a radio-immunoassay capable of detecting nanogram amounts of αFP. These tests have been used jointly to follow the course of patients with αFP positive disease. Serums obtained in collaboration with Dr. C. L. Vogel (Uganda Cancer Institute) have shown that by the time of diagnosis of hepatoma there is a remarkable stability in serum αFP which is not altered by ineffectual chemotherapy and may continue for 2-12 months with only a slight rise. The differences between tumors may extend over a range of 6 logarithms, but each tumor appears to have a characteristic level. Surgical removal of tumor, contrasted to chemotherapy in producing a marked and rapid fall in αFP. The serial determination of αFP in patients with embryonal cell carcinoma and choriocarcinoma have shown a good correlation in decreasing αFP with successful therapy.

In order to correlate serum levels with tumor size (cell number) we are studying the synthesis and catabolism of αFP, initially in monkeys. The half-life of injected αFP is 3.7 days using non-labelled αFP in a normal monkey. Studies are now underway to measure the half-life and fractional catabolic rate of labelled αFP in normal and hepatoma-bearing monkeys. We are also studying the in vitro synthetic rate of αFP production in freshly removed hepatoma tissue. When all details have been worked out in monkeys then the studies will be duplicated in humans and also extended to other αFP synthesizing tumors.

Methods for purification of αFP by immunoprecipitation have been worked out so that αFP from hepatoma, embryonal cell carcinoma, gastric carcinoma and other disease states where elevation occurs. Initial comparisons will look for antigenic differences or two-dimensional peptide may differences after tryptic digestion.

Amino acid sequences of the first 23 amino acids have been determined on 40 mouse K chains. In contrast to human K subgroups (only 4), the mouse appears to have at least 25 (or 50). This implies that the somatic mutation concept must operate on a relatively large number of germ line genes (not one). In contrast to this large variability of K, mouse lambda is extremely restricted; 8 lambda's were found to be identical whereas 4 showed only 1 to 4 mutational differences.

Two myeloma proteins with activity against choline and phorphorylcholine have been shown to have different idiotypes. Only very minimal differences upon sequencing have been found in the variable region that determine differences in serologic determinants. This finding is important for any further

tests by the use of anti-idiotype antisera to determine if variable regions are determined by autosonal genes

By uridine-^{3}H labeling technique, an RNA virus sedimenting at a density of 1.20 gm/ml in sucrose density gradient centrifugation was detected in the supernatant fluid of a culture of lymph node biopsied from a patient with Hogkin's disease. Efforts have been made to examine its infectivity, MSV genome rescue activity, and immunologic reactivity. Preliminary evidence indicates that the virus can act as a helper for MSV genome present in a non-producer cell line. A similar culture from another patient was negative. A third culture is being attempted. It is hoped that findings and techniques gained in SJL/J mouse study may serve as a model for the above investigation.

It is planned to collect more cultures of lymph nodes from Hodgkin's and other disease patients. Serum samples shall be collected and their immunologic reactions against this virus be examined.

In order to understand the natural history of a murine leukemia virus (MuLV) in SJL/J mice and to investigate the etiological relationship of the virus to the Hodgkin's-like reticulum cell neoplasm found spontaneously in high incidence in this strain of mouse, an attempt was made to isolate MuLV from spleens of SJL/J mice of various ages. The virus was found to be N-tropic, antigenically related to AKR-Gross virus, and present in spleens in titers ranging from 10^{1} to 10^{2}. The isolation rate was highest in ages 4 and 8 months, and the titers of naturally occurring neutralizing antibody was found to be higher in these age groups. The virus was used as a helper for rescue of MSV from nonproducer cells and the resultant MSV(SJL) produced a subcutaneous "atypical granuloma" in thymectomized and antilymphocyte serum-treated SJL/J mice. It also produced a lymphoblastic leukemia 5 months after neonatal injection. Immunogenicity of these tumors and viruses in SJL/J mice in terms of cytotoxic antibody and neutralizing antibody production, and protection against spontaneous leukemia and reticulum cell neoplasms is being studied.

Hybrid cell strains that have been made between normal neurons and human fibroblasts do grow and many of them make long processes which resemble the axons and dendrites of neurons. Dr. Minna's previously published work with hybrid cells between the C1300 neuroblastoma of mice and theL cell (mouse fibroblast) had led him to anticipate that most hybrids with neural cells might preserve neuronal properties and fail to show the "extinction of differentiated characters" so often reported with hybrid cells. These putative neuron hybrids showed no unequivocal neuronal properties (electrical activity, acetylcholine esterase, choline acetyl transferase) other than the questionable criterion of process formation. We could conclude that either the normal neurons are not "dominant" or we had not yet made hybrids with differentiated neurons. Our cross was made between rodent neurons and human fibroblasts and we anticipated that the human chromosomes would be lost - this process should return to us cells progressively more and more like the parent rodent neuron as the human chromosomes "sebregated" from the hybrids. Actually, we found that only about two-thirds of the hybrids lose human chromosomes as expected, while one-third lose rat chromosomes. The

rat/man hybrid is much more stable than the mouse/man hybrid; nearly complete complements of both human and rat chromosomes could be found in many of the hybrid cells even after 40-60 cell generations.

Six to eight months after the hybrids were formed, sub-clones which had been isolated from the original rat/man hybrids and some of the mouse/man hybrids have become reduced to 6-10 human chromosomes. At this time we have found the first evidences of the reappearance of the suppressed neuronal functions we are looking for. Acetylcholine esterase activity, though weak, has been unequivocally demonstrated by histochemical staining in 5 different hybrid strains. Choline acetyl transferase activity has been detected in one strain. It now appears that our expectation will be fulfilled and that cells with highly differentiated neuronal properties will appear as the last remaining human chromosomes are lost.

It will be of great importance to be able to produce dividing cells with neural properties from the individual cells of different parts of the nervous system.

Serial No. NCI-3200
1. Laboratory of Cell Biology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Factors influencing the induction and repression of primary tumors and the growth and inhibition of transplantable neoplasms. (Host and tumor cell variables)

Previous Serial Number: Same

Principal Investigator: Dr. Lloyd W. Law

Other Investigators:
Dr. E. Appella (NCI-3229)
Dr. Kenneth Chang (NCI-5550)
Dr. Peter Wright (NCI-5551)
Dr. J. McCoy (Bionetics Research Laboratories, Bethesda, Md.)
Dr. Samuel Strober (Stanford University School of Medicine, Stanford, Calif.)

Man years:
Total - 2 1/2
Professional - 1
Other - 1 1/2

Project Description:

1. Studies of the biologic properties of solubilized, purified transplantation antigens (H-2) and of virus-specific tumor antigens.

Collaborative studies with Drs. Ettore Appella, Samuel Strober and Peter Wright have continued with the following pertinent findings:

a) Using an F2 fraction of solubilized H-2 antigen (specified by the $H\text{-}2^a$ gene) and containing the major $H\text{-}2^a$ specificities, it was possible to show that this material has enhancing properties and also induces immunologic tolerance in appropriate hosts. Soluble antigen-treated CBA and $B.10D_2$ mice grew the allogeneic A strain tumors Sarcoma I and YAA-C1; controls did not nor did those mice receiving the F3 fraction known to contain predominantly non-H2 gene products. Passive transfer of enhancement was also achieved and the response was shown to be specific - that is anti-$H\text{-}2^a$ enhancing antisera did not enhance non-$H\text{-}2^a$ allogeneic tumors.

The biologic completeness of the F2 fraction was also further tested in immunologic tolerance studies. B.10D_2 strain mice were treated from birth, daily, with the F2 fraction and then assayed for their cell-mediated and humoral antibody (cytotoxic and hemagglutinins) responses. The antigen-treated mice retained their capacity to reject B.10A congenic skin grafts but were found to be tolerant to humoral antibody induction. The majority of mice were unresponders and tolerance was found to persist for approximately 150 days after termination of antigen treatment. Immune tolerance was found to be specific in that the treated B.10D_2 mice responded normally to H-2^b antigens (not shared by H-2^a). Preliminary evidence also indicates a striking reduction in enhancing antibodies - as well as reduction of cytotoxins and hemagglutinins.

This study to date therefore strongly indicates that:

1. The F2 fraction obtained by papain digestion and G150 Sephadex chromatography contains all of the major H-2^a determinants (for which it was possible to assay by inhibition of ^{51}Cr release),

2. This fraction contains in addition immunogenic properties - thus directly relating serologic determinants with biologic determinants,

3. The completeness of the molecular structure is assured by the assays of enhancement and tolerance.

Work is continuing to attempt to relate the cell-mediated response with the humoral response and with attempts to describe a possible "blocking" antibody - responsible for the induction of tolerance.

b) Collaborative work continues with Drs. Ettore Appella, Peter Wright and Mark Drapkin on attempts to further characterize the membrane-bound tumor antigens, virus specified VSTA, with the objective in mind of solubilizing and purifying these. Two systems have received attention: tumor (BALB/c) mice adapted to in vivo and in vitro assays; this neoplasm contains strong SV40-specified transplantation antigens. RBL-5, a lymphoid neoplasm, has been shown to be strongly immunogenic and contains tumor antigens of the transplantation type specified by Rauscher leukemogenic virus.

Crude soluble fractions of mKSA tumor cells inhibit specifically the cytotoxicity of anti-mKSA antisera; to date however this fraction has not been shown to be immunogenic in vivo; nevertheless the crude membrane fraction is immunogenic which provides hope that mKSA antigen can be solubilized and purified under proper conditions.

Crude soluble (CS) and, a G-150 Sephadex fraction, F2, similar in composition to the H-2^a F2 fraction, described above, have been shown to provide resistance to the transplantation of RBL-5 leukemic cells. In vitro monitoring of the purification steps are now possible in this system since anti-RBL-5 sera are cytotoxic and the crude soluble and F2 fractions

of antigen inhibit the cytotoxicity. No infective leukemogenic virus has been found in the CS or F2 fraction, strengthening previous observations recorded from this laboratory that these tumor antigens, induced by the RNA viruses of the leukemia-sarcoma complex, are "new cellular" antigens and not structural viral antigens.

Some contamination of our F3 fraction with RBL-5 antigen has been observed so that future efforts will be concerned with more adequately controlled papain digestion of membranes.

2. Role of antithymocyte serum (ALS) in oncogenesis by the RNA leukemia-sarcoma complex viruses.

A striking reduction in the frequency of MSV-induced intramuscular tumors has been achieved by adoptive transfer of MSV antisera as late as 7 days after virus infection, at a time when palpable tumors are present; BALB/c (3 months of age) following ALS are the test animals. Sera from "regressor" syngeneic mice or from hyperimmunized mice (but not from normal or from "progressor" mice) were effective repressors. Though initially there was thought to be a close correlation between effect and titer of neutralizing antibody, this does not now seem to be the case. Adoptive transfer of lymphoid cells though effective in preventing MLV-induced leukemia in identical recipients was not effective in preventing MSV-induced tumors.

Though "blocking factors" are stated to be effective through in vitro analyses, we have been unable to confirm any influence, in vivo, of antisera influencing the capacity of normal or sensitized lymphoid cells to prevent the induction of lymphoid leukemia.

3. Studies of non-releaser neoplasms

Collaborative work continues with Drs. Kenneth Chang (NCI-5550) and Dr. J. McCoy (Bionetics Research Laboratories) on the biologic properties of non-releaser neoplasms induced by the pseudotype viruses (MSV-MLV) and MSV(HLV).

a) XM-1, a hemangiosarcoma, induced initially by MSV(MLV) is a non-releaser neoplasm though originally it contained defective MSV rescuable only by Rauscher LV. This neoplasm was cloned in vitro and the immunogenic character of these clones studied. Assays for defective MSV, infective MSV and of MLV and of anti-MSV or anti-MLV neutralizing antibodies and of the immunosensitivity and immunogenicity of the clones have been accomplished. These studies indicate that clones H.1 and H.14, though neoplastic do not contain infective or defective virus, yet maintain cross-reactivity with the parent strain XM-1 and maintain immunogenic and immunosensitivity characters;

clone H.11 is not neoplastic yet is capable of immunizing. The parent H.11 does not contain infective virus yet a subclone does contain MLV. This has not changed the neoplastic or immunogenic character of H.11. Several of these neoplasms giving no evidence of the presence of MSV or MLV nevertheless do contain RNA-dependent DNA polymerase - indicating presence of virus presumably integrated in the DNA.

b) An anaplastic sarcoma was induced in vitro with MSV(HLV) (Harvey) in PD4 inbred hamster tissue. Following passage in vivo a non-producer clone (PD4 T-1) was selected and the biologic character studied in comparison with the parental "producer" line. PD4 T-1 was shown to contain a tumor antigen that is not an MSV(HLV) virion antigen. Though PD4 T-1 is resisted in hamsters immunized either with homologous virus or with the parent subline (also containing infectious virus), PD4 T-1 is capable of immunizing against itself.

Studies are in progress 1) to adapt PD4 T1 to in vitro analyses in order to compare cross-reactivity with "new cellular" antigens specified by MSV in the mouse and rat and 2) to gain insight into the nature of the virus genetic material apparently still existent in the hamster tumor cell.

Serial No. NCI-3200
1. Laboratory of Cell Biology
2. Office of the Chief
3. Bethesda, Md.

Publications:

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Law, L. W., Appella, E., Strober, S., Wright, P. W., and Fischetti, T.: Induction of immunological tolerance to soluble mouse histocompatibility-2 (H-2) antigens. Proc. Nat. Acad. Sci. (USA). In press.

Honors and Awards:

Named American Association Cancer Research representative to the Division of Medical Sciences, National Research Council

Named NCI Representative for a second three-year term, to the U.S.A. National Committee, International Union Against Cancer.

Serial No. NCI-3229
1. Laboratory of Cell Biology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Chemical structure of immunoglobulin molecules

Previous Serial Number: Same

Principal Investigator: Dr. Ettore Appella

Other Investigators: Dr. R. Mage (NIAID)
Dr. John Inman (NIAID)
Dr. Oliver Roholt, Roswell Park Memorial Inst.
Dr. David Pressman, Roswell Park Memorial Inst.

Man Years:
Total: 2
Professional: 1
Other: 1

Project Description:

1. Mouse immunoglobulins

Two mouse IgA myeloma proteins, MOPC 511 and MOPC 167 have been studied - these myeloma have antibody activity for choline and phosphorylcholine but differ by the idiotypic determinants; it is of interest to define the structural differences in the heavy and light chain variable regions. The two IgA's, purified by affinity chromatography, have been compared for their amino terminal sequence obtained by a Beckman Sequencer, or the tryptic peptides resolved on a fingerprinting map. The first 24 residues of both light chains showed no variations; their tryptic peptide maps differed by a single arginine containing peptide. The first 19 residues of the heavy chains showed an interchange of valine for leucine at position 4, but their maps were identical. Detailed sequence analysis of these proteins is currently underway and attempts will be made to correlate idiotypic determinants and chemical changes of these proteins.

Two mouse lambda chains (HOPC-1 and 511) have been compared in their complete amino acid sequence. No differences were detected, a finding previously reported for two other lambda (MOPC-104 and RPC-20). The total of mouse lambda sequences being identical is now eight and only four showed one to four mutational differences (Weigert et al. Nature 228: 1045, 1970). The results indicated a highly restricted variability in this system and are compatible with predictions of both germ line and somatic theories. The

study of another mouse lambda (SA 15) has been started and should provide an answer in favor of one or the other theory; in a sense that a repeat of one of the variant sequences would argue for a germ line theory, whereas a new variant could be interpreted as a result of somatic mutation.

The amino terminal sequence of the first 23 amino acids of 10 other mouse Kappa chains has been determined; this increases the number of V_K sequences in the mouse to about 40. From these data it appears that the number of V_K subgroups is about 25; a large number indeed when compared to the four V_K subgroups of human Kappa. This value is at the moment a minimum value, first because only the first 23 amino acids were considered and secondly the samples are not random since the repeats or identical sequences are a choice of myeloma proteins with antibody activity.

2. Rabbit immunoglobulins

Various reports of antibodies that appear to have restricted heterogeneity have beenpublished. For example, hyperimmunization of rabbits with streptococcal and pneumococcal vaccines has resulted in a response of limited heterogeneity. We have analyzed the sequences of the peptic and tryptic peptides that contain half-cystines and the N-terminal amino acid sequence of the light chain of a homogenous anti-benzoate antibody of b4 allotype.

Six internal half-cystine sequences were identified and at each position in the amino terminal portion of the molecule, a single major amino acid was obtained in good yield. On the other hand, the light chains of pooled normal IgG of the same allotype was a mixture. The limited heterogeneity of the antibodies found in this case would appear to depend on the stimulation of only a few cells, rather than on a structural identity of the environment around each individual hapten, particularly since the carrier is a heterogeneous mixture of globulins.

Recently, an anti-p-azobenzenearsonate antibody of limited structural heterogeneity was obtained which had light chains of the b5 allotypes. The amino-terminal sequence of this particular light chain and light chains prepared from normal pooled IgG of b5 rabbits was determined by the use of a Beckman Sequencer. This light chain from the restricted antibody of V_{K11} length had a single major sequence in contrast to the b5 light chain from the pool where evidence of structural heterogeneity was detected. The amino terminal sequence of the anti-p-azobenzenearsonate antibody from a b5 rabbit resembled that of anti-benzoate antibody from a b4 rabbit in all but 3 of the first 21 residues, and is evidence that the first 21 residues of rabbit kappa chains are not directly contributory to the antibody specificity regions of the molecules. Idiotypic specificities were not shared in spite of early identical sequences at the N-terminus of two other light chains from

anti-SVIII antibodies (Jaton et al. Biochem. 10, 1583, 1971) and this is a further indication that variabilities at the N-terminus may reflect subgroup relationship instead of antibody specificity.

A search for locating an area of the variable region containing the antibody site has been started. By utilizing iodination techniques (Friedenson et al. J. Immunol. 107, 957, 1971) rabbit antibodies of limited heterogeneity, (anti-p-azobenzoate, anti-3-azopyridine and anti-p-azophenyltrimethylammonium) were labeled and the heavy and light chains separated after mild reduction on Sephadex G-100. The greater portion of the label was at times on the heavy chain (anti-3-azopyridine) and other times on the light chains. Peptide analysis after cyanogen bromide cleavage or tryptic and chymotryptic digestion showed most of the label (a tyrosine residue) to be located in position 100 from the amino terminal end of the variable regions. These studies are now being extended to the mouse myeloma proteins with phosphorylcholine activity (603 and 167). Since both 603 and 167 are readily crystallizable this affords one the opportunity to study the topography of the antibody combing site by relating primary, secondary, and tertiary structure.

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Serial No. NCI-3229
1. Laboratory of Cell Biology
2. Office of the Chief
3. Bethesda, Md.

Appella, E., Chersi, A., Roholt, O. A., and Pressman, D.: Amino acid sequences of light chains of a rabbit anti-p-azobenzoate antibody. Fed. Proc. 31: 1296, 1972.

Rudikoff, Stuart, Hannon, Janet E., and Appella, Ettore: Partial amino acid sequence of two mouse anti-phosphorylcholine myeloma proteins. Fed. Proc. 31: 3897, 1972.

Inman, J. K., Hannon, J. E. and Appella, E.: Demonstration of a simple method for reducing losses of tryptic peptides during automated sequencing. Biochem. Biophys. Res. Comm. 46: 2075, 1972.

Appella, E.: Amino acid sequence of mouse lambda chains. J. Immunol. 107: 930, 1971.

Mage, R. G., Appella, E., Young-Cooper, G. O., Winfield, J. B., Pincus, J. H. and Chersi, A. A rabbit anti-p-azobenzenearsonate antibody of restricted heterogeneity with light chains of b5 allotype. J. Immunol. (in press)

Serial No. NCI-5551
1. Laboratory of Cell Biology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Development of in vitro tests to detect tumor specific transplantation antigens

Previous Serial Number: Same

Principal Investigator: Peter W. Wright, M.D.

Other Investigators:
Dr. L. W. Law (NCI-3200)
Dr. E. Appella (NCI-3229)
Dr. R. Herberman (NCI-5009 c)
Dr. Mark Drapkin (NCI-LCB)
Dr. Wallace P. Rowe (NIAID-LVD)
Dr. Douglas Lowy (NIAID-LVD)

Man Years:
Total - 2
Professional - 1
Other - 1

Project Description:

A cytotoxic assay using ^{51}Cr-labelled target cells has been used to detect an SV40-specific cell surface antigen (TSSA) expressed by various clones of BALB 3T3 cells infected by SV40 and selected in factor-free tissue culture medium. These studies have shown that only those cell lines that are tumorigenic and elicit SV40 specific tumor transplantation resistance in vivo express the SV40 TSSA. These results support our earlier conclusion that the cytotoxic assay is a reliable, quantitative in vitro measure of SV40 tumor-specific transplantation antigenicity.

This technique is currently being utilized in collaboration with Dr. Mark Drapkin (NCI-LCB) to isolate and characterize subcellular soluble SV40 transplantation antigen.

The microcytotoxicity assay (MCA) modified from the colony inhibition test initially devised by the Hellstroms has been utilized in two separate studies:

1. In the first, the MCA was compared with another in vitro measure of cell-mediated immunity, a 51-chromium-release assay (CRA). The two methods were employed concurrently to assess the cellular immune response to a transplanted, syngeneic, Gross virus-induced lymphoma in W/Fu rats. These studies indicated that the information provided by these two assays, tested under a variety of experimental circumstances was essentially equivalent, and that the activities measured by both methods parallel the existing

state of tumor immunity in the intact host, as revealed by standard transplantation tests (in collaboration with Dr. Rinald Herberman, NCI- 5009 c.

2. In the second, the MCA was used to determine the cell-mediated immune response in mice acutely infected with lymphocytic choriomeningitis (LCM) virus, lymphoic cells from mice receiving LCM virus were shown to specifically inhibit the growth of LCM-infected fibroblasts in vitro. The cytotoxic activity of lymphoid cells from mice immunosuppresssed by various means was depressed. These studies indicate that a lymphocyte-dependent, serum complement-independent immune mechanism is of primary importance in the mediation of acute LCM virus-induced disease. These studies have also demonstrated that the MCA can also be utilized to detect murine leukemia virus antigens expressed by non-transformed, virus-infected fibroblasts (in collaboration with Drs. W. P. Rowe and D. R. Lowy, (NIAID-LVD).

Publications:

In vitro relationship of SV40 tumour-specific surface antigen to other SV40 antigens. Nature New Biology, 233: 18-19, 1971.

Serial No. NCI-3222
1. Laboratory of Cell Biology
2. Immunochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Genetic and biochemical mechanisms controlling protein synthesis in well-differentiated neoplastic cells, mouse plasma cell tumors

Previous Serial Number: Same

Principal Investigator: J. Frederic Mushinski, M.D.

Other Investigators:
Dr. M. Potter (NCI-5556)
Dr. P. Periman, George Washington University
Dr. Gunter von Ehrenstein, Max Planck Institute for Experimental Medicine, Göttingen, Germany
Dr. Harold P. Morris, Howard University
Dr. Matthew D. Scharff, Albert Einstein School of Medicine

Principal Investigator: J. Frederic Mushinski, M.D.

Cooperating Units:
Max Planck Institute for Experimental Medicine, Göttingen, Germany
Howard University
Albert Einstein School of Medicine

Man Years:
Professional - 1
Other - 1

Project Description:

A. Mouse IgA Myeloma Proteins - Objective 3 - Approach 2.

The study of the nature of the two-chain IgA immunoglobulin, so far found only in an occasional BALB/c mouse plasma cell tumor, has developed along two lines. The first involves a study of the covalent polypeptide structure of these proteins. To this end tryptic peptides have been isolated from the heavy chains of one such two-chain IgA molecule and from the heavy chain of a more typical polymeric IgA myeloma protein, antigenically and genetically closely related to the two-chain molecule. Utilizing the maximum similarity exhibited by these two proteins, we have been able to identify peptides common to both molecules and those unique to each particular IgA type. Amino acid compositions have been determined on acid hydrolysates

of all these peptides from two different preparations of light and heavy chains of 6A protein (polymer) and 6C protein (two-chain). Six peptides from the common region of mouse K chains have been identified with certainty and several other peptides, presumably from the variable region, have been isolated in major yield and are identical between the light chains of the two proteins. A similar comparison between the heavy chains of the 6C and 6A proteins has been much more difficult. Seventeen tryptic peptides from both 6A and 6C heavy chains seem to have identical composition and amount to about 196 amino acid residues in common between the two heavy chains. Three peptides in large yield were isolated from the 6A protein alone but no major yield peptides unique to 6C heavy chain were isolated. This finding fits with our previous finding that 6A heavy chain was larger than 6C heavy chain, but these 3 peptides do not account for more than 17 of the approximately 80 amino acid residues expected to be found in 6A and not 6C protein. The larger polypeptides were not well resolved in these procedures and must be further studied.

Since the most reasonable location for the polypeptide missing in the shortened heavy chain characteristic of two-chain IgA molecules is toward the carboxyl terminal end, we have been preparing protein from all of the few viable tumors which secrete two-chain IgA's and searching for new ones. Preliminary studies using carboxypeptidase A suggests that all these molecules have heavy chains with C-terminal glutamine whereas polymeric IgA heavy chains usually have C-terminal tyrosine. Whether these two-chain IgA proteins represent another subclass of IgA in the mouse or a genetic mutation leading to a functional heavy chain deletion cannot be determined as yet and requires further study.

The second approach to learning the nature and origin of these two-chain IgA immunoglobulins will involve an attempt to show that such molecules may result from mutational events acting on cells originally synthesizing the more common polymeric-type IgA. We are preparing antiserums which will specifically precipitate only two-chain IgA molecules. With these antiserums we will test clones of polymeric IgA secreting cells from plasma cell tumors and observe whether spontaneous or induced mutational events will result in two-chain IgA producers.

B. Mouse plasma cell tumor transfer RNA. Objective 3 - Approach 1.

We are further pursuing our previous findings that every mouse plasma cell tumor apparently has a different pattern of isoaccepting tRNA's as judged by chromatographic separation on reversed phase chromatography. We have established that these patterns are stable and reproducible phenotypic markers characteristic of the tumor from which the tRNA was isolated. Only two tumors presumed to be genetically related (6A and 6C, see above), has identical tRNA patterns among a large number of plasma cell tumors. We

have grown up tumors isolated as single cell clones from a previously cloned line of MPC 11. These tumor lines, from the laboratory of Dr. Matthew Scharff (Albert Einstein School of Medicine), differ in that one secretes a 7S IgG myeloma protein while the other has completely ceased production of any secretory immunoglobulin. We are also in the process of isolating tRNA from two independently induced K chain secreting plasma cell tumors which secrete K chains identical in amino acid sequence except for 2 residues. We hope to be able to reason from comparison of the patterns of these tRNA's whether tRNA patterns reflect the clonal origin of tumors or reflect the nature of their secretory protein.

C. Rat hepatoma transfer RNA Objective 3 - Approach 1.

We are presently extending the tRNA findings reported in mouse plasma cell tumors by comparing tRNA's from a series of transplantable tumors of a different histological type (hepatomas) in a different species (rat). Three well described hepatomas from the laboratory of Dr. Harold Morris (Howard University) with different degrees of anaplasia have been found to have several differences in amounts of certain isoaccepting tRNA peaks. We are developing new conditions for doing the reversed phase chromatography to repeat our previous, unpublished findings with better peak resolution. We are extending this series by studying RNA isolated from regenerating rat liver at intervals of 1,2,3 and 7 days after partial hepatectomy. It is likely that the process of rebuilding a substantial portion of the resected liver will increase the synthesis of all the protein-synthesizing machinery, and we will see whether tRNA synthesized during this process maintains the same sort of distribution of isoaccepting species as in normal liver or resembles more anaplastic hepatoma.

D. Immunologic responses to plasma cell tumors Objective 6 - Approach 4.

Protective immunity can be established in BALB/c mice such that they are resistant to induction of tumors by a small number of malignant plasma cells. We have begun experiments aimed at investigating the role of the secreted immunoglobulin in this immunity. So far mice repeatedly immunized with a two-chain IgA protein have not shown any protection against challenge with the tumor cells (MOPC 88) producing this protein. Another approach being tried is to see if an xenogeneic antiserum to a secreted light chain can transfer any protection to a challenge of tumor cells from the tumor that secreted the light chain. If conditions can be worked out for some success with this antiserum, experiments in human patients will be considered. To this end certain human K chains are being purified and used to induce a precipitating or cytotoxic antiserum in horses and goats.

E. Induction of plasma cell tumors in rats Objective 1 - Approach 4.

In the hopes of being able to study the etiology and pathogenesis of

plasma cell tumors in animals less commonly infected with leukemia viruses and other viral particles (A-type) we are trying to induce plasma cell tumors in inbred rats. Our initial efforts in this endeavor have been made using the methods which have proved successful in BALB/c and NZB mice, intraperitoneal injections of mineral oil and tetramethyl pentadecane, in six sets of five animals each: inbred male Osborne-Mendel, Wistar, Sprague-Dawley, Fischer, and Buffalo rats and inbred female Sprague-Dawley rats, all from the NIH animal production section. We hope to enlarge the scope of the study to include other inbred rat strains and other carcinogenic treatments such as X-ray and antilymphocyte serum. We have had inquiries from several laboratories in this country anxious to have purified rat immunoglobulins, not currently available since a rat plasma cell tumor system has yet to be developed.

Publications:

Mushinski, M. F.: Stability of chromatographic patterns of amino-akyl transfer ribonucleic acid from individual mouse plasmacytomas and variability among different immunoglobulin A producing plasmacytomas and normal organs. Biochemistry 10: 3917-3924, 1971.

Serial No. NCI-5552
1. Laboratory of Cell Biology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Production and analysis of hybrid somatic cells: their application to some problems of importance in biology and medicine

Previous Serial Number: Same

Principal Investigator: Hayden G. Coon, Ph.D.

Other Investigators:
Dr. George R. Martin (D:LB)
Dr. Igor B. Dawid, Carnegie Inst., Baltimore
Dr. Ivan B. Horak, Carnegie Inst., Baltimore
Dr. John H. Sinclair, Indiana University
Mr. G. David Trisler (C:LCB)
Dr. John D. Minna (H:LBG)
Dr. Peter O. Kohler (NICHD:RPL-1)
Dr. Harry Gelboin (NCI-4766)

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

In last year's annual report we listed six different lines of investigation which were being pursued as research projects in this laboratory: 1) Collagen synthesis in hybrid cells; 2) Somatic Fertilization; 3) Mitochondrial DNA in Hybrid Cells; 4) Chromosome Isolation from Hybrid Cells; 5) Malignancy of Hybrid Cells; 6) Culture of Differentiated Cells (particularly neurons) and their Hybrids. During the past year two of these major projects (#3 and 6, M-DNA and Neuron Hybrids) have matured and are now yielding exciting results. Three of our projects (#1,2,4) have been temporarily shelved and await the anticipated arrival of two post doctoral fellows (May 1 and July 1). One project, #5, the Malignancy of Hybrid cells, has been abandoned altogether. We have determined that we do not now have, nor do we have prospect of having sufficient space or people to permit us to compete with, or even to supplement, the work of the Harris and Klein group. It is our judgement that the projects described in this report can just be fit into our severely limited space/people resources and that in any event they promise results of more far reaching importance for basic biological research and to problems of growth control in mammalian cells than do the Harris and Klein studies.

Serial No. NCI-5552
1. Laboratory of Cell Biology
2. Office of the Chief
3. Bethesda, Md.

1. The Natural History of Mitochondrial DNA in Hybrid Cells: the Beginning of a Somatic Genetics of the Cytoplasmic Organelles of Mammals.

The organelles which have been successfully studied in hybrid cells are the chromosomes. We are interested in studying the cytoplasmic organelles and in the possibility of developing a genetics of their inheritance and interdependence upon the nuclear genome. While we may have leads to some markers for the smooth ER (AHH activity and its product identification profile) as well as the possibility of studying centrioles (basal bodies) in the ciliated ependymal cell cultures we have isolated from rat brain, the mitochondria remain the most promising organelle system for study in hybrid cells. Two recent papers (Clayton, D., R. L. Teplitz, M. Nabholz, H. Dovey, W. Bodmer (1971) Nature, 234; 560-562; and Attardi, B. and G. Attardi (1972) Proc. Natl. Acad. Sci. (Wash.) 69; 129-133) have reported failure of large research efforts designed to isolate and detect the mitochondrial DNA (M-DNA) of both mouse and man from mouse/man hybrid cells. Exaggerated and pompous theories of why the mitochondria must be lost as human chromosomes are lost from the hybrid strains were promulgated. In collaboration with Dr. Igor B. Dawid and Dr. Ivan B. Horak at the Carnegie Institution of Washington, Department of Embryology, Baltimore, we have continued the research project outlined in last year's annual report. Dr. Dawid has developed a method which permits identification of the M-DNA of different species by molecular hybridization (on filters) of the labelled complimentary RNA (made by E. coli polymerase from highly purified M-DNA) to relatively unpurified samples of DNA from cell cultures. Because of the extensive unique sequences of the M-DNAs in different species, this technique may be made sufficiently sensitive to detect about 0.1% human M-DNA in the presence of 99.9% mouse M-DNA and the thousand-fold excess of the nuclear DNAs (N-DNA) of both species. This spring we have obtained good evidence that in numerous independently derived hybrids between rat or mouse neurons and human fibroblasts the M-DNA of both species may persist for many cell generations. This result is different from those reported by Clayton et al. and the Attardis most probably because the rat/man hybrid cells we used tend to retain most of the chromosomes of both species for 60 cell generations or more, whereas the mouse/man hybrids studied by the other two groups tend to have lost most of their human chromosomes by the 40th cell generation. We have recently found, however, that some mouse/man hybrids we have isolated have retained easily detectable quantities of human M-DNA. Furthermore, in certain of our mouse neuron/VA-2, human fibroblast hybrids which are reverse segregants (i.e., have lost mouse rather than human chromosomes) we have found persistence of mouse M-DNA in the presence of no detectable (less than 2%) mouse nuclear DNA. This remarkable persistence in "human cells" of mouse M-DNA, even in the apparent absence of nuclear chromosomal support shows us how well mouse M-DNA competes in mouse/man hybrid cells and "explains" the failure of Clayton et al. and the Attardis to find evidence of human M-DNA in the face of overwhelming amounts of mouse M and N-DNA in their hybrids. Also, our results could mean that the cells used in the cross (mouse neurons x human fibroblasts) and not the species involved could be the significant factor in M-DNA or N-DNA retention or loss.

TABLE 1. ASSAYS OF MITOCHONDRIAL DNA AND NUCLEAR DNA (RAT or MOUSE) IN RAT/MAN and MOUSE/MAN HYBRID CELL STRAINS - EXPRESSED AS % OF TOTAL.

HYBRID CELL STRAIN	% MITOCHONDRIAL DNA	% NUCLEAR DNA
(rat/man)		
VRE 14	14	45
VRE 15	29	47
VRE 17	7	39
VRE 18	20	41
VRE 20	0*	0
VRE 21	19	57
VRE 33	25	48
VRE 41	35	52
VRE 42	7	45
VRE 43	92	50
subclones:		
VRE 43-1	80	60
VRE 43-2	88	54
VRE 43-9	96	33
VRE 43-11	80	60
VRE 47	56	30
VRE 67	2	5
VRE 75	0	0
VSC 13	13	21
VSC 21	83	48
subclones:		
VSC 21-1	98	65
VSC 21-4	75	54
VSC 21-5	70	80
VSC 21-7	95	68
VSC 21-8	85	81
VSC 21-11	88	71
(mouse/man)		
VMB 6	100	70
VMB 10	22	0
VMH 2	100	53
VMH 3	100	62
VME 15	78	10
VMSG 2	100	91
VMSG 6	28	0
VMSG 11	100	65
VMSG 16	20	0
VMSC 10	70	13
VMSC 13	55	0
VMSC 16	99	60
VMSC 21	87	22

*0% rat M-DNA means 100% man M-DNA.

The table lists the results of the analysis of independently isolated hybrid strains and their subclones as per cent (rat or mouse) M-DNA and N-DNA. Note that the N-DNA value represents primarily the rapidly reannealing fraction and is not necessarily correlated with chromosome number. Obviously, the establishment of that correlation has a very high priority in our future work.

The fact that mitochondrial DNA is found in a hybrid cell line after 40 or more cell generations does not necessarily mean that there are cells in the hybrid population which contain both M-DNA of rat and man; it is possible that some of the cells contain rat while others contain human M-DNA and that no cells in the population contain both types. Consequently we made dilute, single cell platings of several strains and picked 20 well isolated, symmetrical colonies from each strain. These colonies in their turn were grown up and assayed (adding still another 15-20 cell generations) and all were found to be mixed with respect to the two species' M-DNA. This finding means that individual cells after some 40 generations in culture as hybrids still produce progeny which contain M-DNA of rat and man and that therefore the cell which formed each colony must have contained replicating M-DNAs of both rat and man.

Some hybrids retain human M-DNA even though they are losing human chromosomes. Karyologic analysis of these hybrids should indicate which human chromosomes (if any) are retained with replicating human M-DNA.

These positive findings are most encouraging and promise a series of important observations on the interrelationships among the M and N-DNAs of mammalian cells. Some first questions are: 1) Can M-DNA of one species replicate (persist) in the absence of the homologous N-DNA? (Some of our man/mouse hybrids appear to say that this is possible.) 2) Does persistent replication of the M-DNA of one species depend upon the presence of specific homologous chromosomes? 3) Are hybrid or "recombinant" M-DNAs formed? 4) Can one species' M-DNA live (or function) in the "skin" of another species' mitochondrion (those structural mitochondrial proteins coded by the nuclear DNA)? 5) What functions besides the syntheses r-RNA and t-RNA of mitochondria are attributable to the presence of the M-DNA of another species?

We have found that these studies need not be limited to heterospecific hybrid cell strains. Surprisingly, we have found that there is a significant difference (about 15% in hybridization experiments) in the mitochondrial DNA between Mus musculus and a subspecies of mice. BALB/c and Mus musculus mollosinus mitochondrial DNA can be distinguished by the c-RNA hybridization procedures used to distinguish the M-DNAs of man and rat. Dare we imagine that it will turn out that each of the inbred strains of mice will be distinguishable one from another? One is tempted to imagine the ghost of Otto Warburg looking over our shoulders when we ask if the M-DNA of tumor cells differs significantly from that of normal cells - especially when analyzed after prolonged animal or in vitro passage.

2. Hybrid Cells between Normal Neurons and Fibroblasts

Nearly from the beginning of cell fusion studies in 1965 it was found by Henry Harris that cells which do not synthesize DNA may resume its synthesis when they are fused with another cell which does synthesize DNA. Harris' classic demonstration of this fact was illustrated by showing that the nucleated chicken erythrocyte could become completely reactivated (lose heteropyknosis, initiate DNA synthesis, and develop functional nucleoli) in the cytoplasm of a dividing cell. We have reasoned directly from Harris' result that it should be possible to derive dividing, hybrid cell strains from specialized cells which have foresworn division during normal ontogeny. Many of these cell types (e.g. neurons, cardiac muscle and plasma cells, erythrocytes) have been urgently sought as cell cultures but have been available for study in cell culture only as rare neoplasms of dubious relation to their normal counterpart. As a result of this past year's work on testing this notion, we have concluded that the production of dividing cell cultures from "terminally" differentiated cell types will soon become one of the most important applications of the cell hybridization technique.

Together with Dr. John D. Minna (NHLI) we have isolated about 250 clonally derived hybrid cell strains between cells of the central nervous system of embryonic rats and mice and human fibroblast cell lines which can be killed in cell culture. We are interested in hybrids between normal neurons and fibroblast lines, however, our starting material is simply a cell suspension derived from retina, optic cortex, spinal cord, and sensory ganglia. These suspensions contain varying amounts of non-neuronal cell types. We have found that many fold purification with respect to neurons may be achieved by taking advantage of the fact that the neurons do not attach to plastic petri dishes as readily as do glial cells and other supporting cells. Our hybrid cell strains made between normal neurons (thus defined) do grow and many of them make long processes which resemble the axons and dendrites of neurons. Dr. Minna's previously published work with hybrid cells between the C1300 neuroblastoma of mice and the L cell (mouse fibroblast) had led him to anticipate that most hybrids with neural cells might preserve neuronal properties and fail to show the "extinction of differentiated characters" so often reported with hybrid cells. However, our putative neuron hybrids showed no unequivocal neuronal properties (electrical activity, acetylcholine esterase, choline acetyl transferase) other than the questionable criterion of process formation mentioned. We could conclude that either the normal neurons are not "dominant" like the C1300 neuroblastoma or we had not yet made hybrids with differentiated neurons. Our cross was made between rodent neurons and human fibroblasts and we anticipated that the human chromosomes would be lost - this process should return to us cells progressively more and more like the parent rodent neuron as the human chromosomes "segregated" from the hybrids. Actually, we found that only about two-thirds of the hybrids lose human chromosomes as expected, while one-third lose rat chromosomes. The rat/man hybrid is much more stable than the mouse/man hybrid; nearly complete complements of both human and rat chromosomes could be found in many of the hybrid cells even after 40-60 cell generations.

Within the last month, 6-8 months after the hybrids were formed, subclones which had been isolated from the original rat/man hybrids and some of the mouse/man hybrids have become reduced to 6-10 human chromosomes. At this time we have found the first evidences of the reappearance of the suppressed neuronal functions we are looking for. Acetylcholine esterase activity, though weak, has been unequivocally demonstrated by histochemical staining in 5 different hybrid strains. Choline acetyl transferase activity has been detected in one strain. It now appears that our expectation will be fulfilled and that cells with highly differentiated neuronal properties will appear as the last remaining human chromosomes are lost.

We do not yet know whether all of the human chromosomes may be lost from a hybrid of this kind and still leave a propagating neuron-like cell strain. At the least we may come to know which human chromosome must be present to cause continued division in neuron and we shall be able to determine if it is the same one required for division of other cell types like cardiac muscle. This information about the specific human chromosomes concerned with growth control in different normal tissues may be expected to complement and extend the kinds of data being sought by Klein and Harris and the group with Leo Sachs as to which chromosomes are present or absent in malignant cells and their hybrids. It will also be interesting to note whether all of the propagating neuron-like cells obtained from our hybridizations grow in the parent mouse strain like malignant cells.

No immunological reagents are known which are specific for particular types of neuronal cells. Class specific reagents exist which have been prepared from proteins so abundant that they may be purified directly from the nervous system (S-100 protein of glia and possibly the 1546 protein from neurons, as well as anti-myelins found in M.S. patients). But the immunology of nerve cells per se, in spite of its great promise, has been thwarted because individual cell types could not be isolated in pure form or in sufficient quantity to yield high titre antisera of diagnostic specificity. A graduate student in our laboratory, Mr. G. David Trisler (Indiana University, Bloomington) has undertaken to develop antisera (in rabbits) directed against the surface components of several of our most promising segregated rodent neuron/human fibroblast hybrids. After cross adsorbtion (in vitro and in vivo), the specificity of these reagents may easily be tested against the normal neurons of the region from which the hybrid's parent cell came. It may happen that hybrid cells may be used to explore the mysteries of the specificities of individual neurons by immunochemical methods.

It will be of great importance to be able to produce dividing cells with neural properties from the individual cells of different parts of the nervous system. Just as it was important 10 years ago to produce (and identify as such) homogeneous cell strains producing specific immunoglobulins - the mouse plasmacytomas - so it will be important to produce (and characterize) homogeneous strains of particular neurons. The ultimate goal of this work is to develop

cell strains which will make possible the exploration of the molecular basis of neuronal individuality and synaptic specificity.

3. Projects near completion:

A) Aryl hydrocarbon hydroxylase induction in hybrid cells.

Dr. F. Wiebel and Dr. H. Gelboin (NCI) have assayed a rather large variety of hybrid cell combinations (both heterospecific and homospecific) between cells which show induction of Aryl hydrocarbon hydroxylase (a membrane associated enzyme system mediating the carcinogenesis of aromatic hydrocarbons) They found that such combinations yielded hybrid cells which were not inducible just as Schneider and Weiss have already reported for another famous "inducible" enzyme system (tyrosine aminotransferase, TAT). Most hybrids work this way:

$TAT^{ind+} \times TAT^{ind-} \rightarrow TAT^{ind-}$ and AHH similarly: $AHH^{ind+} \times AHH^{ind-} \rightarrow AHH^{ind-}$

However, one combination yielded a dramatically different result, A9 (an 8-azaguanine resistant "L" cell derivative) by a BUdR resistant, 3T3 mouse embryo fibroblast line: 3T3-4C2. In that cross:

AHH^{ind-} (mouse, A9) $\times$ AHH^{ind+} (mouse, 3T3-4C2) $\rightarrow A9/C2\,AHH^{ind+++}$

The details of this observation are sufficiently interesting to warrant a separate publication which is now in preparation. Future work with this system may take advantage of the fact that the A9/C2 hybrids are "epithelioid" and are "contact inhibited" unlike either parent cell population. Segregant clones from these A9/C2 hybrids which are not contact inhibited are expected to be $AHH^{ind\pm}$ or AHH^{ind-} (more like the A9 parent). Quite obviously, this system will prove intriguing to evaluate for possible suppression of malignancy especially in view of the fact that A9 is the cell line common to the most convincing of the Harris and Klein studies. One wonders how good the correlations will be among the AHH inducibility, the state of contact inhibition, and malignancy of these and other hybrids involving A9 and/or 3T3-4C2. Our interest in these hybrids extends as well to the fact that we may have a useful entrée into a marker system for one of the membrane systems which is regulated in hybrid cells.

B) JEG-3 is a human choriocarcinoma cell line developed by Dr. Peter Kohler (NICHD). It was an attractive candidate for hybridization

because of its multiple markers of specialized synthesis: estradiol (E), progesterone (P), and choriogonadotrophin (HCG). A number of hybrid lines with mouse and human cells were prepared. The E, P, and HCG syntheses of the JEG-3 parent line appears to have been "extinguished" in the mouse/human hybrids. However, several hybrid lines between JEG-3 and a human fibroblast line, VA-2 are especially interesting because of their continued evidence of HCG synthesis, i.e.:

$$(man)\text{JEG-3}^{E^+}, P^+, HCG^+ \quad \times \quad \text{VA-2}^{E^-}, P^-, HCG^- \ (man)$$

$$\downarrow$$

$$\text{JEG-3/VA-2}^{E^-}, P^-, HCG^+$$

This result implies that VA-2 may not exert "negative control" over HCG synthesis and also suggests that each of the hormones is separately regulated in the hybrid. This is only the second example of a multiply marked cross which has been analyzed. Evidence for coordinate control of specialized synthesis in multiply marked cells is being sought in several laboratories because of the importance such evidence will have in exploring the mechanisms of cellular differentiation. Abstracts have been submitted and after more karyological analysis can be done, a paper will be forthcoming.

Publications: None

Serial No. NCI-5553
1. Laboratory of Cell Biology
2. Immunochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structure of mouse myeloma proteins with antibody activity

Previous Serial Number: None

Principal Investigator: Dr. Stuart Rudikoff

Other Investigators: Dr. Michael Potter (NCI-5556)
Dr. Ettore Appella (NCI-3229)
Dr. David R. Davies (NIAMD-7)

Man Years: Professional - 1

Project Description:

A series of mouse myeloma proteins with various antibody activities has been developed in this laboratory by Dr. Michael Potter (NCI-5556). We are currently involved in two major studies with these proteins which are designed to elicit information concerning the structure of the antibody molecule.

1. In collaboration with Dr. Ettore Appella (NCI-3229) we are continuing work on the determination of the primary amino acid sequence of two proteins, MOPC 511 and MOPC 167 which have activity to phosphorylcholine and are beginning studies on several other proteins with antibody activity.

2. In collaboration with Dr. David Segal, Dr. Eduardo Padlan, and Dr. David Davies (NIAMD-7) we have succeeded in crystallizing the combining site of two myeloma proteins with activity to phosphorylcholine, McPC 603 and MOPC 167. The McPC 603 crystals give a high degree of resolution (2.7°A) in preliminary X-ray defraction studies. Studies are in progress to determine both the primary and tertiary structure of this molecule which should produce the first three dimensional model of an antibody combinint site. Attempts to crystallize the clmbining sites of other myeloma proteins and to determine their primary structure are in progress.

It is felt that studies of this nature will lead to the eventual in vitro design and synthesis of antibody molecules with determined specificities. These molecules would be of an extreme diagnostic as well as possibly therapeutic value.

Serial No. NCI-5554
1. Laboratory of Cell Biology
2. Immunochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: A. Characterization and measurement of soluble antigens associated with human neoplasms

B. Pathogenesis and pathophysiology of plasma cell neoplasms in mice and characterization of their immunoglobulin products

Previous Serial No.: Same

Principal Investigator: K. Robert McIntire, M.D.

Other Investigators: Dr. Philippe P. Sizaret, I.A.R.C., Lyon, France
Dr. Charles L. Vogel, Uganda Cancer Institute, Kampala, Uganda
Dr. Thomas A. Waldmann (NCI-4002)
Dr. Richard Adamson (NCI-3509)

Cooperation Units: I. A. R. C., Lyon, France
Uganda Cancer Institute, Kampala, Uganda

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

A. Characterization and measurement of soluble antigens associated with human neoplasms. Objective 6 - Approach 1.

1. Studies on Alpha-Fetoprotein (αFP). Recent reports using sensitive radio-immunoassay methods have shown that αFP no longer is associated exclusively with hepatocellular carcinoma and embryonal cell carcinoma, but is elevated in a wide variety of neoplasms and liver disease and is actually present in normal adult serum in ngm/ml amounts.

a. Quantitation of αFP: Extending the work completed with Dr. Philippe P. Sizaret (I.A.R.C.) of developing a simple quantitative test for αFP we have now used these reagents to set up a radio-immunoassay with Dr. T. A. Waldmann (NCI-4002) capable of detecting nanogram amounts of αFP. These tests have been used jointly to follow the course of patients with αFP positive disease. Serums obtained in collaboration with Dr. C. L. Vogel (Uganda Cancer Institute) have shown that by the time of diagnosis of hepatoma there is a

remarkable stability in serum αFP which is not altered by ineffectual chemotherapy and may continue for 2-12 months with only a slight rise. The differences between tumors may extend over a range of 6 logarithms, but each tumor appears to have a characteristic level. Surgical removal of tumor, contrasted to chemotherapy in producing a marked and rapid fall in αFP. The serial determination of αFP in patients with embryonal cell carcinoma and choriocarcinoma have shown a good correlation in decreasing αFP with successful therapy. A contract for radio-immunoassay of αFP has now been set up and will supply much needed information regarding incidence of elevation of αFP in a wide variety of neoplastic diseases.

b. Metabolism of αFP: In order to correlate serum levels with tumor size (cell number) we are studying the synthesis and catabolism of αFP, initially in monkeys in collaboration with Dr. Richard Adamson (NCI-3509) The half-life of injected αFP is 3.7 days using non-labelled αFP in a normal monkey. Studies are now underway to measure the half-life and fractional catabolic rate of labelled αFP in normal and hepatoma-bearing monkeys. We are also studying the in vitro synthetic rate of αFP production in freshly removed hepatoma tissue. When all details have been worked out in monkeys then the studies will be duplicated in humans and also extended to other αFP synthesizing tumors.

c. Physicochemical studies of αFP: Methods for purification of αFP by immunoprecipitation have been worked out so that αFP from different hepatomas may be chemically compared. Comparison may also be made of αFP from hepatoma, embryonal cell carcinoma, gastric carcinoma and other disease states where elevation occurs. Initial comparisons will look for antigenic differences or two-dimensional peptide map differences after tryptic digestion

2. Studies of Lung Tumor Associated Antigens: Objective 5 - Approach 4. Recently there has been much investigation concerning a carcinoembryonic antigen (CEA) associated with cancer of the colon, first described by P. Gold and co-workers. While the absolute specificity of the antigen is in some doubt it has obvious potential as a biochemical marker of tumor activity, similar to αFP. We have immunized 21 rabbits with a variety of extracts from various normal and malignant pulmonary tissue. After absorption with normal lung extracts, 3 antisera appear to have some ability to discriminate malignant tissue extracts. One of these has been studied more thoroughly and has been shown to be identifying an antigen different from CEA, α2H-ferroprotein, β-fetoprotein and α1-fetoprotein. It was found to be positive with 20 of 28 lung tumor extracts. When tested against extracts of malignant tissue other than lung there was a positive reaction with melanoma and 4 sarcomas (Burkitt's, Reticulum Cell, Osteogenic and Rhabdomyosarcoma). The only extract of normal tissue giving a positive reaction was spleen. No serum sample from lung tumor patients was positive. Physicochemical characterization of this antigen has been started. A Bronchiogenic carcinoma maintained in tissue culture by Dr. A. Rabson has been shown to synthesize

the antigen and we are presently labelling the antigen with ^{14}C-amino acids to be used as a marker for following purification of the protein.

Current work is directed at purification of the antigens and production of higher titer antiserum and these will be used to develop a quantitative assay. Serologic testing might then be available for clinical screening.

B. Mouse immunoglobulins.

1. Immunoglobulin markers on plasma cell surface: Object 3 - Approach 5. Studies performed in collaboration with Mr. Gerald Princler using the cytotoxic effect of heterologous antiserum specific for IgF showed that 6/7 tumors of this class contain IgF on their cell surface. Plasma cell tumors secreting other classes of immunoglobulins do not show this cytotoxic effect or are very weakly positive. In contrast to IgF synthesizing cells, IgG plasma cells are lysed when complement is added and this lysis can be blocked by heterologous antiserum against mouse IgG, indicating the presence of host antibody on the surface of the tumor cells. IgG cells grown in tissue culture by Mr. D. Trisler do not have the surface immunoglobulin, but when the cells are returned to in vivo growth they again demonstrate IgG on the surface. Work in progress is designed to elucidate the functional role of plasma cell surface Ig markers.

2. Objective 3 - Approach 4. IgA levels in mice during plasma cell tumor (PCT) induction: Serial IgA levels have shown a 2-3 fold rise in IgA after mineral oil or Pristane, IP. This level rises most rapidly near the time of PCT development and may be helpful in studying the physiology of the plasma cells in the population at risk. These levels will also be compared to levels of other Ig classes during the carcinogenesis process.

Publications:

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Shelton, E. and McIntire, K.R.: The ultrastructure of the γM immunoglobulin molecule. J. Mol. Biol. 47: 595-597, 1970.

Leon, M.A., Young, N.M., and McIntire, K.R.: Immunochemical studies of the reaction between a mouse myeloma macroglobulin and dextrans. Biochem. 9: 1023-1030, 1970.

Sizaret, P.P., McIntire, K.R., and Princler, G.L.: Quantitation of human alpha-fetoprotein by electroimmunodiffusion. Cancer Res. 31: 1899-1902, 1971.

Takahashi, T., Old, L.J., McIntire, K.R., and Boyse, E.A.: Immunoglobulin and other surface antigens of cells of the immune system. J. Exp. Med. 134: 815-832, 1971.

Serial No. NCI-5554
1. Laboratory of Cell Biology
2. Immunochemistry Section
3. Bethesda, Md.

McIntire, K.R., and Vogel, C.L.: Quantitative studies of alpha-fetoprotein during progression of hepatoma. Second Conf. and Workshop on Embryonic and Fetal Antigens in Cancer. In Anderson, N.G. and Coggin, J.H. (Eds.): Div. Tech. Information, Extension, AEC, in press, 1972.

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Serial No. NCI-5556
1. Laboratory of Cell Biology
2. Immunochemistry Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Pathogenesis of plasma cell neoplasia in mice; immunochemical characterization of antigen-binding myeloma proteins; genetic basis of immunoglobulin diversity

Previous Serial Number: Same

Principal Investigator: Michael Potter, M.D.

Other Investigators:
Dr. R. Asofsky (I:LGAR-116c)
Dr. R. Lieberman (I:LCI-15c)
Dr. S. Rudikoff (NCI-5553)
Dr. David R. Davies (A:LMB-7)
Dr. C.P.J. Glaudemans (A:LC-7)
Dr. A. Nisonoff (Univ. of Illinois)
Mr. David McKean (Univ. of Wisconsin)
Dr. Don W. Bailey (Roscoe B. Jackson Lab., Bar Harbor, Maine)
Dr. Philip Baker (I:LGAR -119a)

Cooperating Units: None

Man Years:
Total: 4
Professional: 1
Others: 3

Project Description:

I. Pathogenesis of Plasma Cell Tumor Development.

Plasma cell tumors can be induced in 60 to 80% of BALB/c mice by the intraperitoneal injection of various mineral oils or pure hydrocarbons (e.g. pristane). Thus far, only mice of the inbred BALB/c and NZB strains are susceptible. Most of the commonly used strains are resistant (e.g. C57BL, C3H, AL/N, DBA/2). The biologic basis for susceptibility is one of the main problems in this laboratory. The restricted ability to induce tumors in only a few specific inbred strains suggests a germ line transmitted genetic factor or factors may be involved in plasma cell tumor susceptibility. In addition other interacting influences play an important role; these are the natural microbial flora and an abnormal tissue environment, the mineral oil conditioned peritoneum.

Serial No. NCI-5556
1. Laboratory of Cell Biology
2. Immunochemistry Section
3. Bethesda, Md.

A. Genetic factors in mineral-oil induced plasmacytoma formation.

1. Linkage of susceptibility to the regulation of the immune response.

It is now well known that many facets of the regulation of the immune response differ among the inbred strains e.g. 1) ability to respond to specific antigens; 2) serum levels of different immunoglobulin classes, notably IgA; 3) cellular proliferative responses vary in degree; and 4) ability of certain types of antigenic preparations to induce tolerance. The BALB/c and NZB plasmacytoma susceptible strains have been implicated as having unusual reactions in some of these parameters in past studies by other investigators. In order to link specific forms of regulation to plasma cell tumor susceptibility it is necessary to have available a series of genetically different test strains. This is a potential first step in identifying an important phenotypic character and a necessary prerequisite to identifying specific genes. An ultimate experimental model system would be to develop a congenic strain of mice that is susceptible and resistant. While this goal is still remote we are very fortunate in having seven new inbred strains of mice developed by Dr. D. W. Bailey of the Jackson Lab., Bar Harbor. These strains were developed from seven pairs of (BALB/c × C57BL/6)F_2 hybrids. Each strain contains a random mixture of genes from the susceptible BALB/c and resistant C57BL strains. We have established these strains in the laboratory and have preliminary data on their susceptibility to plasma cell tumor development by mineral oil.

7 Inbred Strains, Derived Randomly from Pairs of (C57BL/6 × BALB/c)F_2 by D. W. Bailey, Jackson Lab.

Strain	IgC_H	PC.1	H-2	Plasma Cell Tumors (Total)
C × BD	B	C	C	2/14
C × BE	B	B	B	0/32
C × BG	C	B	B	4/15
C × BH	B	B	C	0/36
C × BI	B	B	B	0/13
C × BJ	C	B	B	9/22
C × BK	B	C	B	0/29

C = BALB/c B = C57BL/6

The next phase of this work to be initiated will be to characterize aspects of immune regulatory mechanisms to determine if there is a correlation. In collaboration with Dr. Philip Baker we are investigating the proliferative responses to antithymocyte serum. In BALB/c these responses are 6-fold greater than in C57BL. Further Dr. Baker has found that thymus suppressor functions in BALB/c mice are less effective than in C57BL. Assays will be made to determine if suppressor action of thymus cells is depressed in the strains that are susceptible to plasmacytoma development.

The serum IgA levels of these strains and their response to mineral oil treatment will also be investigated.

2. Linkage to control of somatic mutation.

In man Xeroderma pigmentosum (an inherited disorder associated with the increased susceptibility to develop skin tumors following exposure to UV light) is associated with a biochemical defect in the repair of UV damaged DNA. Hypothesizing that BALB/c mice have some form of defect that interferes with their ability to repair somatic mutations, we proposed last year that mutagens might increase the incidence of plasmacytomas. Three mutagens were tested for their ability to enhance plasmacytoma formation in mineral oil treated mice: N-methyl nitrosourea, fractionated total body x-irradiation and ethylmethanesulfonate (EMS). In the first experiment it was found that nitrosourea induced the formation of a number of lymphoreticular neoplasma thus obscuring a specific ability to increase plasmacytoma development. X-irradiation caused a delay in the appearance of a somewhat decreased number of tumors and probably is not a useful agent because of the possibility of having immunosuppressive action which is known with chemical immunosuppressant to decrease the incidence of plasmacytoma development. EMS which is known to be a highly effective mutagenic agent in the mouse was found to accelerate plasmacytoma development and also to increase the incidence of tumors. Two additional experiments are now nearing completion that confirm this initial observation which is summarized in the table below. The mechanism of action of EMS is not known. We had Dr. Wallace Rowe test this compound for its ability to release infection MuLV virus in his system and he found that unlike Iododeoxyuridine it was ineffective. All this would suggest that EMS may be acting as a somatic mutagen. Further characterization of the EMS effect is underway.

Effect of Ethylmethanesulfonate on Plasma Cell Tumor Induction by 3 Intraperitoneal Injections of Mineral Oil

Exp.	Treatment	EMS No.	220mg/Kg day post first M.O. given	No. mice PCT total	%	day
I	none			24/40		
	EMS	2	83,142	28/46		
II	none			9/32	27.9	367
	EMS	4	64,79,93,107	14/31	47	367
III	none			5/27	18.5	341
	EMS	4	33,48,62,76	7/24	29.1	341
	EMS	4	96,110,123,133	11/24	45.3	341

3. Linkage to Viral Proliferation.

It is becoming increasingly apparent that the mouse as a species carries several viruses belonging to the murine leukemia virus complex (MuLV). As these agents are relatively ubiquitous it becomes more and more difficult to demonstrate their all or none presence or absence. The exciting developments from the work of Rowe, Hartley, Boyse, and Old suggest that the germ line transmitted MuLV as proposed by Gross may actually be due to an incorporation of viruses into the murine genome. If so, the potential carcinogenic activity of such agents may be due to the ability of these viruses to propagate in somatic cells. It is known that somatic cells can be superinfected with MuLV virus even though it is intrinsically produced. The initial experiments planned in collaboration with Dr. L. W. Law have been to attempt to increase plasmacytoma formation in mice given only two intraperitoneal injections of mineral oil with viruses. In the first experiment now nearly complete Moloney Leukemia virus was given to 4-month old mice and the incidence of tumors was compared with mice given only virus and mice given oil. No specific enhancing effect was noted. Many of the mice developed leukemia which obscured the interpretation of the experiment.

B. The role of a natural microbial flora in plasmacytoma formation.

Germfree BALB/c mice treated with mineral oil develop plasma cell tumors only rarely and then usually at an advanced age. In collaboration with Dr. Richard Asofsky (I:LGAR-116c), we have found that BALB/c mice monocontaminated with Salmonella tel aviv survive moderately well under germfree conditions. The Salmonella tel aviv strain was chosen because it had been previously

observed that 2 myeloma proteins specifically precipitated with the O-specific antigen produced by this species of Salmonella. Despite the fact that a number of the mice monocontaminated with Salmonella tel aviv developed unusual illnesses months after the initiation of the monocontamination which unfortunately resulted in the loss of many of these animals before autopsies could be done, we nonetheless were able to observe a slightly increased incidence of plasma cell tumors. During the course of these experiments, however, it became apparent from other publications and our experiences with monocontamination that monocontamination such as we had initially performed was not the best means for studying antigenic stimulation in germfree BALB/c mice. It was considered that antigen feeding would be more effective. Heremans and colleagues in Belgium have shown that feeding of antigens to germfree mice results in a predominantly IgA type immune response. We are currently initiating an experiment in germfree mice in which various antigens will be fed during the latent period. One of the principal aims of this work is to find the method for inducing tumors that produce antigen-binding myeloma proteins to the antigen used to stimulate the germfree mouse. This is deemed important because it will permit us to trace the natural history of the neoplastic plasma cell from its normal progenitor.

Although the experiment is still in early progress a slightly increased incidence of plasmacytomas has developed in mice fed the Trichoderma extract. None of the tumors thus far has produced a myeloma protein that reacts with the extract.

These studies have been extended to include pathogen-free mice. Arrangements were made with Mr. Howard Bohner of the Lab Aids Division to establish a new pathogen free BALB/c mouse strain contaminated with Lactobacillus, Streptococcus D and Bacteroides. These mice have been maintained continuously in tanks and have been given three injections of pristane (either of 1 or of 2 months). Plasmacytomas are just developing.

The results of all of these experiments have suggested that possibly a changing microbial flora may be essential to provide enough stimulation of the IgA lamina propria system of cells so as to make available a sufficient number of precursor cells for subsequent plasmacytoma development. Continuation of these studies is planned.

C. The role of the abnormal peritoneal environment in plasmacytoma development.

1. In the last year we have initiated a complete series of studies on the transplantability of primary plasmacytomas into normal mice and mice that have previously received one or two intraperitoneal injections of mineral oil. Of the first 15 tumors tested in this study 14 have grown rapidly in the mineral oil conditioned mice and only two have grown in normal mice. Cell doses ranging from 10^5 to 10^2 cells were given and 10^2 and 10^3 cells have

been effective in transmitting the tumors. These results dramatically indicated that the conditioned peritoneum either provided special factors that favored plasma cell tumor growth or that because of some alteration in physiology permitted plasma cell tumor growth e.g. by providing a protected environment. Further experiments are planned to elucidate the basis for this selective growth; also attempts will be made to study the proliferation of normal immunoglobulin producing cells in the conditioned peritoneum.

2. Plasma Cell Tumor Antigens.

Whisson and Connors in 1965 reported that mice bearing large grafts of Adj PC5 (5 to 7 grams) could be completely regressed by analine mustard and further that a large percentage of these mice were immune to challenge with AdjPC5 cells. We have attempted to confirm this observation and to identify the nature of the tumor antigen in particular to determine if it is related to immunoglobulin idiotypes or to the PC1 antigen of Takahashi et al. Miss Jamie Walters, 3rd year undergraduate student of the University of Md., a part-time worker in the laboratory, is carrying out this work under my supervision. We have found that mice bearing AdjPC5 can be cured as reported and that mice which have regressed large grafts can resist challenge with AdjPC5 cells. Mice immunized with other tumors have not yet produced immunity to AdjPC5, suggesting that there are several types of antigens.

Immunity to BALB/c Plasmacytomas Induced by Analine Mustard Regressions of Large Tumors

Immunizing Tumor	Challenge Tumor	Cell Dose	No. Mice Positive		
			Control	One Regression	Two Regressions
AdjPC5	AdjPC5	10^5	11/12	12/42	1/14
AdjPC5	AdjPC5	10^6	9/9	2/12	
AdjPC5	AdjPC5	10^5	8/8	15/37	
LPC1	AdjPC5	10^5	8/8		8/8
TEPC15	AdjPC5	10^5	8/8		5/8
XRPC3	AdjPC5	10^5	8/8	8/8	
603NP	603NP	10^5	8/8	10/16	
LPC1	LPC1	10^5	8/8		9/10

Thus far all experiments have been carried out with subcutaneous grafts. Experiments are planned to test intraperitoneal graft and to then determine if immunized mice which have previously received intraperitoneal injections of mineral oil can reject the challenges.

II. Immunochemical Characterization of Antigen Binding Myeloma Proteins.

1. Identification of specific ligands for antigen binding myeloma proteins.

The mouse has been a rich source of homogeneous immunoglobulins with antigen binding activity. Some 16 proteins are under study in this laboratory that have binding activity for a variety of different polysaccharide moieties, phosphoryl choline and nitrophenyl groups. For many of these the specific chemistry of the ligand is known but for others only an antigen has been identified and it becomes essential to find the chemical nature of the determinant group. In collaboration with Dr. C.P.J. Glaudemans (A:LC-7) we are preparing various mono- and oligosaccharides from antigens and testing them as inhibitors in specific systems. Work has been completed on identifying highly active inhibitors for two new myeloma proteins SAPC-10 and TEPC 191; the active inhibitors are oligosaccharides containing 2, 3 or 4 beta 1→6 linked D-galactose. We are currently attempting to establish the binding constant for the di- and trisaccharides. Further insoluble immunoabsorbants have been prepared which makes it possible to purify these proteins in a single step.

Studies are underway to identify the specific oligosaccharide inhibitors for the MOPC384 and McPC870 proteins that both precipitate with the lipopolysaccharides of Salmonella tel aviv and S. tranoroa. Attempts will be made to learn more about the chemical structure of these two polysaccharides.

Collaborative studies continue with Dr. E. A. Kabat, Columbia University on the specificity of MOPC 406 for antigens containing terminal N-acetyl-D-mannosamine. Attempts will be made this year to isolate active oligosaccharides from the polysaccharide. Essentially it is somewhat easier to identify a terminal monosaccharide group but considerably more difficult to find the neighboring sequential chemical groups and this requires (particularly with antigens derived from the Enterobacteriaceae where the polysaccharides contain mixtures of different sugars) chemical study of the polysaccharide itself. Unfortunately many of the mouse myeloma proteins do not react with the characterized common types of Salmonella O-antigens.

Work is in progress on identifying the active chemical ligand in the Hemophilus influenzae type B polysaccharide. The SAPC 15 an IgG, lambda myeloma protein binds this polysaccharide.

2. Studies on crystallization of antigen binding myeloma proteins.

In collaboration with Dr. S. Rudikoff, Dr. David Segal and Dr. David R. Davies (A:LMB-7) we are attempting to isolate in pure form all of the active myeloma proteins, prepare pepsin Fab fragments and attempt crystallization. In the last year D. Givol of the Weizmann Institute successfully

crystallized the MOPC 315 protein and following his technique of using pepsin Fab fragments we have successfully crystallized the McPC 603 myeloma protein using the ammonium sulfate method. These crystal have been shown to have high resolution in X-ray diffraction work resolving out to at least 2.6 angstroms. Accordingly Dr. Davies is collecting full data on the crystal structure and we are determining the primary structure of the 603 Fab in order to build a 3-dimensional model of the immunoglobulin molecule and determine the structure of the active site.

III. Genetic Basis of Antibody Specificity.

1. Study of the complex IgC_H locus.

In the last several years, in collaboration with Miss Rose Lieberman (I:LCI-15c) we have produced myeloma-specific and allotypic antisera by immunization of other strains of mice with BALB/c myeloma proteins of the IgA and IgG classes. These antisera aid in the characterization of identifying specific gene products from the complex IgC_H locus. Understanding of the structure and regulation of this complex locus progresses at this time by comparative studies of homologous loci in other strains. Because plasmacytomas cannot be induced in other strains a complete collection of homogeneous proteins has not been available as a source of antigens for the preparation of allotypic antisera.

Congenic strains are now past the 20th backcross and may be considered to be essentially congenic with BALB/c except for the insertion of foreign heavy chain (IgC_H) complex: BALB/c × C57BL/KaIgC_H BC20 F_2 and BALB/c × AL IgC_H BC20. We have now completed the development of congenic strains of mice which provide a source of myeloma proteins directed by loce other than the one found in BALB/c.

A series of plasmacytomas have been induced in BALB/c × ALIgC_H BC9 F5 by Mrs. Elizabeth Mushinski and these are being used as a source of myeloma proteins for a new series of allotypic studies in collaboration with Miss Rose Lieberman. We are continuing a search for all types of Ig from the C57BL IgC_H complex locus. In over 50 tumors studies we have been unable to recover a myeloma carrying the IgC_H (2) determinant. This result suggests that the gene controlling this protein is not regulated within the congenic host.

2. Study of the complex IgV_H locus.

These strains are being used in collaborative studies with Dr. Al Nisonoff, University of Illinois, in searching for an idiotypic marker that is linked to allotype i.e. and IgV_H gene. Initial studies have been promising in this respect and linkage of an antiarsonate idiotype to the ALIgC_H locus has been found.

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1. Laboratory of Cell Biology
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3. Studies on the IgV_{κ} complex locus.

In the last year in collaboration studies with Dr. Lee Hood (Cal Tech) and Mr. David McKean (Graduate Student, Johns Hopkins University) we have shown that the kappa chains in BALB/c mice have a remarkable degree of variability within the first 23 residues from the amino terminus. At the present time, over 18 subgroups have been delineated among approximately 30 chains so far examined. Several of these sequences were also determined by Dr. Ettore Appella (NCI-5551). This unique property of BALB/c kappa chains not found in the kappa chains of any other species so far, permits subgrouping kappa chains from a readily accessible peptide, mainly the amino-terminal Cys 23 peptide. All unblocked BALB/c kappa chains can be characterized by amino acid sequence determination on the Beckman Amino Acid Sequencer. Currently, in collaboration with Mr. David McKean, we have gone on to explore the complete sequences of 4 proteins which possess the same amino-terminal Cys 23 peptide, e.g. MOPC 70, MOPC 321, MOPC 63 and TEPC 124. Mr. McKean has developed methods for isolating the peptides of this group of kappa chains and has established a tentative primary structure for the sequences of MOPC 321, MOPC 63 and TEPC 124. The MOPC 70 chain differs from all the others in 17 positions along the chain. The MOPC 63 differs from T124 and M321 at 8 positions. The T124 and M321 differ from each other at only 3 positions. These results raise the interesting question of whether it is likely that there will be a germ line gene for each of these minor variants. Still plausible is the possibility that there exists a mechanism for generating diversity at the somatic level using as a basis a pool of germ line genes. Further analysis of this interesting group of kappa chains will be continued by David McKean in Dr. Oliver Smithies laboratory. We will collaborate in supplying them with kappa chains for further sequence work.

Publications:

Baumal, R., Potter, M., and Scharff, M.D.: Synthesis, assembly and secretion of gamma globulin by mouse myeloma cells. III Assembly of the three subclasses of IgG. J. Exp. Med. 134: 1316-1334, 1971.

Potter, M.: Antigen binding myeloma proteins in mice. Ann. N.Y. Acad. Sci. 190: 306-321, 1971.

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Serial No. NCI-5009 (c)
1. Lab. of Cell Biology
2. Cellular & Tumor Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immune Response to Tumor Specific and Other Cell Surface Antigens

Previous Serial Number: NCI-5009(c)

Principal Investigator: Ronald B. Herberman, M.D.

Other Investigators: Chou-Chik Ting, M.D., Joel Engelstein,M.D. Robert Oldham, M.D., Manuel Ortiz de Landazuri, M.D., Marcia Cleveland, M.D.

Cooperating Units: Acute Leukemia Service, Medicine Branch, NCI; Viral Leukemia and Lymphoma Branch, NCI; Uganda Cancer Institute, Kampala, Uganda; George Washington University, Contract PH69-2176; Bionetics Research Laboratories, Contracts PH69-2160, 70-2207 and 72-3227; Laboratory of Immunology, NIAID; Laboratory of Microbiology, NIDR; Drug Evaluation Branch, NCI.

Man Years:

Total:	9
Professional:	5
Other:	4

Project Description:

Objectives: The objectives of this project are: 1) to detect tumor specific antigens and quantitative changes in normal antigens on malignant cells; 2) to detect antibodies and cellular immune reactions to tumor specific antigens; 3) to evaluate the diagnostic, prognostic and therapeutic significance of humoral and cellular immune response to tumor antigens; 4) to detect delayed hypersensitivity reactions and other immunological reactions to human tumor antigens; and 5) to isolate and characterize tumor antigens and other cell surface antigens.

Methods Employed: Sera and cells are obtained from patients and control subjects. A variety of normal and tumor cell lines are maintained in tissue culture. Inbred strains of mice and rats, and transplantable tumors induced by viral, chemical or

leukemic patients. The cytotoxicity assay has also been useful in the study of cellular immunity to tumor antigens in lymphosarcoma, with reactions seen against tumor cells but not against normal cells of the same patients.

c. In a cooperative study with the Acute Leukemia Service, NCI, patients have been skin tested before and after experimental therapy with BCG. No apparent effect of this manipulation on skin reactivity to tumor extracts has been noted.

d. The IUdR cytotoxicity assay is being used to study reactivity of patients with melanoma and breast cancer to tissue culture lines derived from tumors of the same type or of different types. Some tumor specific reactivity has been observed.

e. The lymphocytes of almost all normal individuals have been found to have cytotoxic activity against human lymphoid cell lines in the ^{51}Cr release assay. Patients with cancer or immunosuppression have decreased or no activity. Studies are in progress to determine the nature of this reactivity.

Significance to Biomedical Research and the Program of the Institute:

Detection of tumor antigens and of immune reactions to tumor specific antigens is essential for progress in tumor immunology. The sensitive assays for humoral and cell-mediated immunity which have been developed in the laboratory have already proved useful in studying the immune response to animal and human tumors.

The ability of our laboratory to perform multiple assays of humoral and cell-mediated immunity to tumor antigens should allow detailed dissection of the role of the immune response in limiting or preventing tumor growth. These assays may also be useful in immunodiagnosis of cancer and in measuring changes in immunity, in response to chemotherapy or immunotherapy.

The available immunological techniques have also allowed progress in the solubilization and isolation of tumor-associated cell surface antigens. Such studies will permit a comparison of the physicochemical nature of tumor antigens and normal histocompatibility antigens and may provide material for active immunization of animals and tumor patients against their tumors.

Proposed Course of Project:

Studies are in progress to further study the immune responses of experimental animals and of patients to tumor associated antigens.

Shevach, E., Herberman, R., Lieberman, R., Frank, M.M. and Green, I.: Receptors for immunoglobulin and complement on human mouse leukemias and lymphomas. J. Immunol. 108:325, 1972.

Bluming, A.Z., Ziegler, J.L., Fass, L. and Herberman, R.B.: Delayed hypersensitivity reactions to autologous Burkitt lymphoma protein extracts:Results of a prospective two and a half year study. Clin. Exp. Immunol. (in press), 1972.

Herberman, R.B.: Cellular immunity to human tumor associated antigens. Israel J. Med. Sci. (in press), 1972.

Hollinshead, A.C. and Herberman, R.B.: Separation of the major histocompatibility antigens from other antigens present on human leukemic and white blood cell membranes. Proc. Fifth Internat. Sympos. Compar. Leukemia Res. (in press), 1972.

Herberman, R.B. and Rosenberg, E.B.: Cellular cytotoxicity reactions to human leukemia associated antigens. Proc. Fifth Internat. Sympos. Compar. Leukemia Res. (in press), 1972.

Ortiz de Landazuri, M. and Herberman, R.B.: Immune response to Gross virus-induced lymphoma. III. Characteristics of the cellular immune response. J. Nat. Cancer Inst. (in press), 1972.

Engelstein, J.M., Herberman, R.B. and Waltman, S.R.: Protection of penetrating corneal allografts from immune rejection. Am. J. Ophthal. (in press), 1972.

Ting, C.C., Lavrin, D.H. and Herberman, R.B.: Antibodies to fetal antigens. Proc. Second Conf. and Workshop on Embryonic and Fetal antigens. (in press), 1972.

Rosenberg, E.B., Herberman, R.B., Levine, P.H., Halterman, R., McCoy, J.L. and Wunderlich, J.R.: Lymphocyte cytotoxicity reactions to leukemia associated antigens in identical twins. Int. J. Cancer (in press), 1972.

Herberman, R.B.: Immunological aspects observed in childhood malignancies; rationale for immunotherapy of these tumors. Proc. Radiotherapy Sympos. Clinical Pediatric Oncology. (in press), 1972.

Ortiz de Landazuri, M. and Herberman, R.B.: Specificity of cellular immune reactivity to virus-induced tumors. Nature (in press), 1972.

Ting, C.C., Lavrin, D.H., Shiu, G. and Herberman, R.B.: Expression of fetal antigens in tumor cells and their relationship to tumor specific antigens of papova virus-induced tumors.

to the PC-1 antigen described by workers at Sloan-Kettering. The sera also contain antibodies to a viral envelope antigen (VEA) of viruses found in plasma cell tumors. This VEA is distinct from that associated with Gross virus. Evidence suggests that the observed immunological reactions may be related to a new C-type virus, associated with plasma cell tumors.

k. Cell surface receptors for complement component C3 and for immunoglobulins have been shown to be present on bone marrow derived lymphocytes and absent on thymus derived cells. A large number of mouse leukemias and lymphomas were tested for these receptors, as markers for the origin of the tumor cells. Only one of the tumors appeared to be bone marrow derived. In contrast, when similar studies were performed on human lymphoid tissue culture cells and on chronic lymphocytic leukemia cells, positive results were obtained. The receptors were absent on human acute leukemia cells.

II. Tumor Immunity in Man:

a. Patients with neoplastic diseases have been shown to have delayed hypersensitivity reactions to extracts of tumor cells. Delayed skin reactivity in patients with acute leukemia has been found to correlate with a remission status of the patients. Several patients have become skin test negative when they relapsed and tests became positive again when they went back into remission. Allogeneic skin tests have given some positive reactions, indicating the presence of some common antigens in acute leukemia. Studies have been performed with Drs. A. Hollinshead and C. Alford (George Washington University) on patients with carcinoma of the colon and breast and with melanoma. Colon tumor extracts contain a carcinoembryonic type of antigen which elicits skin reactions; this antigen has been shown to be distinct from the carcinoembryonic antigen of Gold. In melanoma, soluble antigens of two different sizes have been identified. Reactivity to the higher molecular weight antigen appears to correlate with stage of disease.

b. Extensive in vitro studies are being performed in our laboratory on cellular immune reactions to human tumor associated antigens. The short-term ^{51}Cr release assay has been used for study of cellular reactivity to human leukemia associated antigens. Cytotoxic activity was found when the lymphocytes of patients with acute leukemia were tested against viable frozen autologous blast cells. The incidence of reactivity has been 40%; there has not been a significant correlation of reactivity with clinical state. Allogeneic reactivity has also been detected with the assay. The lymphocytes of some patients, family members, and unrelated controls have reactivity against target cells from

In experimental animals, an emphasis will be placed on in vitro studies of cellular and humoral immune responses and correlation with in vivo resistance to tumors. The tumors being studied include those induced by polyoma virus and SV40, Gross and other murine leukemia viruses, murine sarcoma virus and mammary tumor virus. Mouse plasma cell tumors and tumors induced by chemical carcinogens will also be studied. The kinetics and specificity of the cellular and humoral immune responses to tumor associated antigens in these tumors will be studied further. The relationships between the detected antigens and viral gene expression and expression of fetal antigens will be examined. Cells and sera shown to be reactive in the in vitro assays will be used in immunotherapy and immunoprophylaxis experiments.

Studies of human tumor associated antigens and immune response to them will be pursued. Studies on delayed hypersensitivity reactions to tumor antigens associated with carcinomas of the breast, colon and lung, melanomas and leukemia are in progress. In vitro studies on cellular immune reactivity in patients with acute leukemia and melanoma are now being performed, and studies with carcinoma patients are being set up. Reactivity in different assays will be compared and correlations made with the clinical state of the patients. These assays are being used to monitor patients with leukemia who are receiving immunotherapy with BCG or with allogeneic leukemic cells.

An attempt is also being made to study antibodies to human breast cancer by the isotopic antiglobulin technique. Sera obtained from patients at various intervals will be tested against autologous and allogeneic tumor cells in tissue culture.

Honors and Awards:

Elected Fellow of the American College of Physicians.

Publications:

Herberman, R.B.: Serological analysis of cell surface antigens of murine virus-induced tumors. J. Nat. Cancer Inst. 48:265, 1972.

Ting, C.C., Lavrin, D.H., Takemoto, K.K., Ting, R.C. and Herberman, R.B.: Expression of various tumor specific antigens in polyoma virus-induced tumors. Cancer Res. 32:1, 1972.

Rosenberg, E.B. and Herberman, R.B.: Serum factors in renal carcinoma. Lancet 2:1153, 1971.

the Takasugi-Klein microcytotoxicity method (as modified by the Hellströms). These two assays seem to be measuring similar events; many of the differences observed could be accounted for by the in vitro activation phenomenon noted above. Studies are now in progress to compare these two assays with the IUdR cytotoxicity method.

e. The effects of a variety of manipulations on the cellular immune response to Gross leukemia have been studied. Rats inoculated with Gross leukemia virus at birth were shown not to be tolerant to Gross virus-induced surface antigens, but rather developed cellular immunity to these antigens. Thymectomy, treatment with anti-lymphocyte serum, and x-irradiation only partially suppressed the cellular reactivity.

f. The lymphocyte mediated cytotoxicity assay has also been successfully used to study the cellular and humoral immune response to inoculation with Moloney sarcoma virus (MSV). Reactivity was found to vary considerably with the lot of virus used and with the strain of the recipients. In general, cellular reactivity reached a peak at 14 days after inoculation, whereas cytotoxic antibody appeared later and persisted for a longer period.

g. An inhibition technique has been developed in our laboratory for studying the specificity of the cellular cytotoxic reactions. This technique has already provided information on the specificity of the reaction against Gross leukemias in rats and against MSV in mice. The specificity of the cellular reactions are different from the specificities of antibodies. Antigens have been detected on cells transformed by MSV, which do not produce any infectious virus. This provides strong evidence that this specific cell surface antigen is distinct from virus envelope antigen.

h. It has been shown that RNA leukemia viruses can induce a complex of cell surface antigens rather than a single specific antigen. Evidence indicates that rats recognize a broader range of cell surface antigens than mice do.

i. Soluble Gross virus-induced cell surface antigens have been studied, by cytotoxic inhibition and by immunofluorescence. Soluble antigens, with differing specificities, have been found in the sera of mice bearing a transplantable Gross tumor. Large amounts of antigens could be extracted from tumor cells by repeated washing with saline.

j. The sera of most mouse strains contain natural antibodies to antigens on plasma cell tumors in BALB/c mice. One of the antigens detected in these sera has been shown to be identical

unknown agents are used in model systems. Antigen - antibody reactions are measured by complement- dependent cytotoxicity reactions and by an indirect ^{125}I-labeled antibody technique. Cellular immune reactions are measured by skin tests, mixed lymphocyte cultures, migration inhibition, and lymphocyte-mediated cytotoxicity assay. Human and animal tumor and normal cell membrane and soluble fractions are prepared for antigenic and biochemical studies. Tumor cells and normal lymphocytes are tested for membrane receptors by immunofluorescence and by rosette formation with sheep erythrocytes.

Major Findings:

I. Tumor Immunity in Animal Models:

a. The isotopic antiglobulin technique, an indirect ^{125}I-labeled antibody technique, which was developed in our laboratory, has proved useful in extensive studies of the tumor specific cell surface antigens (TSSA) of polyoma virus and SV40 induced tumors. A close correlation has been observed between the TSSA and the tumor specific transplantation antigen (TSTA). This technique has also been useful in studying the relationship of fetal antigens to TSSA and TSTA. Fetal antigens have been found on a variety of mouse tumor cells, but these appear to be distinct from the tumor specific antigens.

b. The cellular immune response to Gross virus-induced cell surface antigens has been extensively studied in a transplantable Gross leukemia in W/Fu rats. The immune reactivity of lymphocytes from the spleen, lymph nodes, peripheral blood and peritoneal exudate became undetectable 20 days after tumor cell inoculation. Evidence indicates that the reactivity is suppressed by some factors in the environment of the host. Lymphocytes, which on immediate testing have no cytotoxic activity, become strongly positive after in vitro incubation for 12-24 hours. This in vitro activation is temperature dependent and requires the presence of a nylon-adherent cell.

c. A new assay for cell-mediated cytotoxicity for monolayer tissue culture cells has been developed in our laboratory. Cytotoxicity is measured by the release of ^{125}IUdR from labeled target cells. This has proven useful in the study of the immune response to Gross leukemia in rats and to human tumor associated antigens.

d. The relationship of various assays for cellular immunity with each other and with the in vivo resistance to tumor have not been clearly defined. In the Gross leukemia system a study has been performed to compare the results of tests with the short-term ^{51}Cr-release cytotoxicity method with the results of

Proc. Nat. Acad. Sci. (in press), 1972.

Shevach, E.M., Herberman, R., Frank, M.M. and Green, I.: Receptors for complement and immunoglobulin on human leukemic cells and human lymphoblastoid cell lines. J. Clin. Invest. (in press), 1972.

Herberman, R.B.: In vivo and in vitro assays of cellular immunity to human tumor antigens. Fed. Proc. (in press), 1972.

Hollinshead, A., McWright, C., Alford, T. and Glew, D.: Separation of skin-reactive intestional cancer antigen from the carcinoembryonic antigen of Gold. Science, (in press), 1972.

Serial No. NCI-5558
1. Lab. of Cell Biology
2. Cellular & Tumor Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immune Response to Tumor Specific and Other Cell Surface Antigens.

Principal Investigator: Chou-Chik Ting, M.D.

Cooperating Units: Laboratory of Biology, NCI; Laboratory of Viral Diseases, NIAID; Bionetics Research Laboratories, Contract PH70-2207.

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: The objectives of this project are: 1) To study the relationship of the tumor specific cell surface antigen (TSSA: detected by the isotopic antiglobulin technique) of papova virus-induced tumors to the tumor specific transplantation antigen (TSTA), and the function of viral genome in regulation of the expression of these antigens. 2) The expression of fetal antigens in tumor cells and their relationship to the tumor specific antigens in papova virus-induced tumors, and their possible application in immunodiagnosis or immunoprophylaxis. 3) Detection of tumor associated antigens in human breast carcinoma.

Methods Employed: Tumor cells transformed by oncogenic virus, chemicals or unknown agents are kept in tissue culture or as transplantable tumors in mice or rats. The antibodies reacting to the TSSA or fetal antigens of these tumors are measured by the isotopic antiglobulin technique. The TSTA was determined by immunorejection test.

Major Findings:

I. TSSA of papova virus-induced tumors:

1. Relationship of TSSA to TSTA: In polyoma virus-induced tumors, it was found that the expression of TSSA correlated

with TSTA. A polyoma cell line which has more TSSA as measured by the isotopic anti-globulin technique also has more TSTA as measured by the immunorejection test (4). In SV40-induced tumors, the SV40 TSSA was also only demonstrated in the SV40 transformed cells which have the TSTA. These results indicated that TSSA and TSTA are closely related. They may be the same antigen. Since both are tumor specific and are located on the cell surface.

2. Function of viral genome: In the SV40 experiments, the "cryptic transformants" revealed some very intriguing nature of the malignant transformation of cells. They have the complete copy or copies of viral genome integrated into the cells, but most of their functions were repressed: they can synthesize T antigen, they can produce tumors in vivo but show contact inhibition in vitro. When tested for TSTA or TSSA, neither was detected. It indicates that the genes which code for these antigens are not expressed. There must be a regulating mechanism which controls the functions of the genes.

II. Fetal antigens in tumor cells and their relationship to tumor specific antigens of papova virus-induced tumors:

The sera which reacted specifically with the TSSA of polyoma (APS) or SV40 tumors (ASVS) did not react with fetal tissue cells and the activities of these sera could not be removed by the absorption with large quantities of fetal tissue. This indicates that fetal tissues do not have detectable amounts of antigens which will cross-react with the TSSA of papova virus-induced tumors (2). In contrast, the antisera produced against fetal tissues will react with various tumor cells in addition to fetal tissue cells, and the activities of these sera against polyoma or SV40 tumors could be removed by absorption with various nonpolyoma or nonSV40 tumors. Therefore, our findings indicate that fetal antigens are expressed in cells after malignant transformation, but they are different from tumor specific antigens which are specific for a particular tumor or tumors induced by a particular virus (5,6).

III. Tumor associated antigens in breast carcinoma:

By using the cell line, F-230, derived from a patient with breast carcinoma (Old et al), the reactions obtained with the sera from patients with breast carcinoma seemed to be more related to HL-A antigens than to truly tumor specific or tumor associated antigens. It may be due to the absence of specific antibodies in these sera, or the F-230 cells do not have the tumor specific or tumor associated antigens.

Significance to Biomedical Research and the Programs of the Institute:

The TSSA of papova virus induced tumors detected by th isotopic antiglobulin technique is shown to be closely related to the TSTA tested by immunorejection experiments. This isotopic antiglobulin technique is a simple, rapid and sensitive assay for tumor specific cell surface antigens, which should be able to be used in human tumors, for diagnostic purposes, or as a monitoring index for immunotherapy, or evaluation of patient's clinical condition.

The re-expression of fetal antigens in various tumor cells may also be useful for the detection or therapy of cancer. Though fetal antigens are not the same as tumor specific antigens, they may still function as a transplantation antigen. If they are present in considerable amounts in certain tumors, they may be used for diagnosis or immunotherapy of these tumors.

The findings of differences in the expression of viral genome in different SV40 transformed cells may offer a clue to the search for the regulating mechanism which governs this genetic function. It may further help to reveal the mystery of malignant transformation of cells.

Proposed Course of Projects:

1. Functions of viral genome in transformed cells:

The direct relationship of viral genome to tumor antigen synthesis and oncogenesis is transformed cells has not yet been definitely established. We plan to study the functions of viral genome in different transformed cells and various mutants, to help to solve these problems.

a. To study the transcription of viral genome in papova virus transformed cells:

Analysis of the viral specific DNA and RNA of standard transformants and mutants, and their relationship to the expression of various tumor antigens and oncogenesis.

b. Purification of tumor specific antigens:

To study the molecular structures of these antigens should help to establish their relationship to viral genome or host genome.

2. Fetal antigens:

The present study has been expanded to analyze the specificities of fetal antigens in various tumor cells and various fetal tissues, and to determine whether only some specificities are reexpressed after malignant transformation, (oncofetal antigen?), and their usefulness in immunotherapy or immunoprophylaxis.

3. Tumor associated antigens in breast carcinoma:

a. Autochthonous system: establish short term or long term tissue culture from breast carcinoma and control with normal breast tissue or skin fibroblast. Sera obtained at various intervals before or after surgery will be tested with these cells.

b. Allogeneic system: Test the sera obtained from patients with breast carcinoma with various established cell lines derived from breast carcinoma.

c. Documentation of these cell lines: morphology, cytogenetics and biochemical analysis.

Honors and Awards:

Elected Fellow of the College of American Pathologists.

Publications:

1. Ting, C.C. and Herberman, R.B. Inverse relationship of polyoma tumor specific cell surface antigen to H-2 histocompatibility antigens. Nature (New Biology) 232:118-120, 1971.

2. Ting, C.C., Lavrin, D.H., Shiu, G. and Herberman, R.B. Tumor specific cell surface antigens of papova virus-induced tumors and their relationship to fetal antigens. Proc. First conf. and workship on Embryonic and Fetal antigens in cancer. 1971.

3. Herberman, R.B., Ting, C.C. and Lavrin, D.H. Immune response to virus induced leukemia in animals immunized with fetal tissue. Ibid, 1971.

4. Ting, C.C., Lavrin, D.H., Takemoto, K.K., Ting, D.C. and Herberman, R.B. Expression of various tumor-specific antigens in polyoma virus-induced tumors. Cancer Res. 32:1-6, 1972.

5. Ting, C.C., Lavrin, D.H. and Herberman, R.B. Antibodies to fetal antigens. Proc. Second Conference and Workshop on Embryonic and Fetal antigens in Cancer, 1972 (in press).

6. Ting, C.C., Lavrin, D.H., Shiu, G. and Herberman, R.B. Expression of fetal antigens in tumor cells and their relationship to tumor specific antigens of papova virus-induced tumors. Proc. Nat. Acad. Sci. 1972 (in press).

Serial No. NCI-5526
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cell surface changes in normal, DNA virus transformed and spontaneously transformed tumorigenic mouse cell lines.

Previous Serial Number: Same

Principal Investigator: Peter T. Mora

Other Investigators: Mrs. V. W. McFarland, Dr. Paul W. Kent, Guest Worker, MBS, LCB, NCI. Dr. Roscoe O. Brady, Jr., Dr. Federico A. Cumar, Dr. Peter H. Fishman, LNC, NINDS; Dr. Richard W. Smith, I., NCI; Dr. K. K. Sanford, LBGY, NCI; Dr. J. P. Bader, Ch., Etiol., NCI; Dr. R. Bassin, VLL, Etiol., NCI.

Cooperating Units: Department of Pathology, N.Y.U., School of Medicine.
Bionetics Research Laboratory, Bethesda, Md.
Department of Biochemistry, Oxford University, Oxford, England.

Man Years:

Total:	5 1/2
Professional:	3 1/2
Other:	2

Project Description:

Objectives: A defect occurs upon viral transformation of cells in the completion of the carbohydrate chains in the glycolipids, especially in those glycolipids (the gangliosides) which contain sialic acid at terminal position. Another change occurs in cell surface glycoproteins, also containing sialic acid. The objective was to clarify the biochemical mechanism of both of these changes and relate these changes to cell growth differences in tissue culture and also in vivo. A further objective was to study the effect of halogenated sugars as modifiers of cell surface sialic acid, and probably concurrently modifiers of cell transplantability

and immunogenicity.

Methods Employed: Tissue culturing methods (humidified CO_2 incubator, roller bottles, suspension cultures, etc.). Labelling of gangliosides, and glycoproteins, also of nucleic acids and proteins, etc., with radioactive precursors during cell growth in culture. Cell homogenization and subcellular fractionation techniques such as isolation of cell membranes. Chemical isolation and characterization procedures, including thin-layer chromatography and gas-liquid chromatography. General enzymology techniques. Radioactivity counting procedures. Tumor take studies in mice.

Major Findings: I. Glycolipids. a) Changes in DNA Virus Transformed Established Cell Lines. In Swiss mouse cell lines after transformation with SV40 or polyoma virus, there was shown to be a reduction in the activity of an enzyme (uridine diphosphate N-acetylgalactosamine:hematoside N-acetylgalactosaminyltransferase) necessary for the synthesis of the higher gangliosides. These changes were found to be reversibly coupled to the changes in growth properties in tissue culture:in the flat derivatives of the virus transformed cell lines which were selected out (by R. Pollack, Cold Spring Harbor Laboratory) by an FUDR treatment as revertants to normal phenotypic growth properties in culture, there was a trend of reversion in ganglioside pattern and in enzymatic activity near to that of the parent 3T3 line, in spite of the continued presence of the viral DNA.

Mouse cells productively infected with polyoma virus did not show decrease in the aminosugar transferase activity. Cocultivation of virally transformed cells with enzymatically active cells did not result in reduced enzyme activity in the enzymatically active cells, or in the restoration of enzyme activity in the virally transformed cells. Similarly, no change in enzyme activity occurred when admixing homogenates of the two type of cells.

Similar results were obtained in Balb/c cell lines transformed by SV40 and in certain "flat" derivative cell lines selected out by H. Smith and C. Scher by a differential need for serum growth factor requirement. The "flat" cells continue to carry the viral genome but are subject to contact inhibition of growth in culture, and show also the reversal in the transferase activity and in higher ganglioside content. The enzyme depression does not occur in lytic, virus-producing infection, when the whole virus genome is being transcribed and translated. Thus the enzyme depression appears to pertain specifically to the viral function(s) involved in cell transformation. But insertion of the virus genome into the cell genome (and continued expression of the T antigen) is not sufficient: the "flat revertant" cells can be looked upon as variant cells which are capable to repress stably the growth

property alteration by the inserted virus genome. Significantly, these cells also depress the depression of the transferase. Thus the activity of the transferase is controlled by cells conjointly with the functioning of that part of the virus genome which (possibly together with a part of the cellular genome into which it is inserted) seems to be instrumental in the maintenance of the transformed growth property in culture.

The main conclusion from this work is that when the functioning of a certain part (other than the complete T-antigen) of SV40 or polyoma viral genome appears to be necessary for the maintenance of the altered growth properties of the virally transformed cells, its functioning may be expressed or repressed by the cell carrying viral genes parallel to the expression or repression of a very specific biochemical event. The biochemical event, which in the flow of information is distal to DNA, involves the activity of at least one key transferase enzyme necessary for the synthesis of membrane glycolipids. The biochemical control of the N-acetyl-galactosamine transferase activity change is yet unknown. The observations up to date are consistent with at least a partially common repressor-like mechanism in both SV40 and polyoma virus-induced transformation of growth properties of mouse cells in culture.

b) Biochemical Mechanism. Several enzymes, necessary for the stepwise biosynthesis of higher gangliosides, were compared in normal (Swiss 3T3) and in virally transformed derivative cell lines (SV40 or PY Swiss 3T3).

The only drastic change found was the above described decrease in the N-acetyl-galactosaminyl transferase. Neither DNA or RNA or protein biosynthesis inhibitors altered selectively the enzyme activities. No restoration of enzyme activity (or phenotypic growth property in culture) was observed upon adding concentrated N-acetylgalactosaminyl transferase preparations to cells in culture.

In an SV40 transformed Balb/c clonal cell line, which has no detectable viral function at all (clones obtained from H. Smith) but contain viral DNA, there was no change in the enzyme activity as compared to the parent clone (Balb OA31).

Temperature sensitive mutant cell clones from Swiss 3T3, (collaborative work with C. Basilico, N.Y.U.),do not show coordinate modulation of enzyme activity (or serum growth factor dependence) while modulating the cell saturation density when shifted between permissive and non-permissive temperatures.

c) Generality of Glycolipid Changes:Direct Effect of DNA Viral Transformation or Indirect Effect Due to Selection During Transformation? Since only a small percentage of cells become

permanently transformed upon infection with a DNA virus, it is possible that only those cells have been selected out to transform which already had low enzyme activity. From various mouse strains (AL/N, Balb/c, Swiss) when testing numerous established cell lines (3T3, 3T12, etc.), clonal derivative lines and polyoma or SV40 transformed derivative cell lines and clonal virus transformed cell lines, we have found one exception in 26 independently virus transformed cell lines (including about 12 clonal lines) to the general finding. Specially designed clonal studies are in progress and no final conclusion can be reached as yet.

d) RNA Virus Transformed Cells. The Swiss 3T3 cell line was carefully titrated with a Moloney sarcoma virus (MSV) to find the conditions of transformation which results in essentially 100% of cells being morphologically transformed in about 48 hours. There were no changes observed 48 hours after transformation, however, after 96 hours the N-acetylgalactosaminyl transferase activity decreased to 50%, and after seven days to 20% of the original activity. This type of transformation kinetic experiments avoid the dual problem of selection and of effect of long range continuous cultivation (see below). Previous less controlled work in established cell lines (Balb/c, Swiss, AKR) and in their MSV or KiSV (Kirstein sarcoma virus) transformed derivative lines, revealed no reproducible differences.

e) Non-Viral Factors in Glycolipid Changes. Prolonged continuous cultivation (over two years) was observed on two occasions to lead to spontaneous loss of higher gangliosides. The reason for this is under investigation. Certain cells (L cells) which were cultivated in serum free medium for a very long time, also are deficient in higher gangliosides, but in a few weeks recover some of the gangliosides when shifted to a medium containing fetal calf serum. Minor changes in medium (Eagles vs. Dulbecco Vogt), pH, tricine buffer, etc., the density of cells at harvest, the growth rate of cells, etc., do not affect significantly the glycolipid composition or the enzyme activities in numerous cell lines investigated by us.

f) Resistance of Glycolipid Changes to Selective Pressures. SV40 transformed cells in their syngeneic host (Balb/c or AL/N) may induce tumor especially if the immunological capacity of the host is impaired. Cells grown out from such tumors were established in culture. Several such in vivo passage and re-establishment of cell lines did not alter the decreased transferase activity. Thus, in established transformed cell lines in vivo or in tissue culture selection pressures do not operate against the viral induced biochemical change.

II. Glycoproteins. a) Effects of Modifiers of Glycoprotein Biosynthesis on Aminosugar Metabolism in Cultivated Cells.

(Collaborative work with Dr. P. W. Kent, Oxford University). Two mouse cell lines T AL/N and SVS AL/N have been grown for one generation (ca 16 hrs.) in 0.5 μM to 5 mM N-fluoroacetyl-D-glucosamine without detectable toxic effects. Below 0.2 mM, the fluoro-sugar modifier increases the incorporated $^{14}C/^{3}H$ ratio for whole cells of both lines with [1-^{14}C]D-glucosamine and [G-^{3}H]L-threonine as labels. The ratio is substantially diminished at concentrations above 0.2 mM by comparison with modifier free controls. Isolated glycolipid and glycoprotein fractions from cells grown in the presence of N-gluoroacetylglucosamine (0.5 mM)have specific activities appreciably lower than control fractions. Growth of cells for 6 days under these conditions, however, leads to elevation of the specific activities of the glycoprotein fraction. N-Iodoacetylglucosamine exhibits similar short term effects but it is more toxic. Parallel studies have been made of the effect of modifiers on [^{3}H]L-threonine, [^{3}H]thymidine and [^{3}H]uridine incorporation in double labelling experiments with ^{14}C-glucosamine. Evidence indicates that the SVS AL/N cell line synthesizes more glycoprotein than the T AL/N cell line. The action of the halogeno-sugars appears to be due at least in part to selective modification of aminosugar metabolism.

b) Effects of Modifiers of Glycoprotein Biosynthesis on Cell Surface Sialic Acid and on Tumorigenicity (Transplantability) of Cells. Growth of both T AL/N (spontaneously transformed AL/N mouse cell) and SVS AL/N cell lines for six days under 0.5mM N-fluoroacetylglucosamine led to a significant increase (about ten-fold) in the incorporation of [1-^{14}C]-D-glucosamine into high molecular weight glycoprotein fraction, obtained after papain digestion of cell homogenates followed by dialysis of the digest. When the cells were grown under similar conditions and were recovered in intact form, and by a careful V. cholera neuraminidase treatment only the cell surface sialic acid was removed, it was found that the neuraminidase accessible cell surface sialic acid in the terminal position of the glycoproteins was also greatly enhanced. T AL/N cells grown under 0.5 mM N-fluoroacetylglucosamine were found transplantable and produced lethal tumors in the allogeneic Balb/c mice. Untreated T AL/N cells do not induce tumor in Balb/c mice. This indicates that modification of some of the histocompatibility antigens in the T AL/N cells can be attained by the sugar modifiers.

Significance to Biomedical Research and the Program of the Institute: Studies of cell surface biochemical changes which relate to loss of contact inhibition in culture and to acquisition of tumorigenic potential in vivo are of interest, especially when they may lead to understanding of changes in molecular mechanism (enzymology) which appears to be reducible to heritable changes on control processes on the DNA level in the virally induced transformation of cells.

Proposed Course of the Project: There will be an attempt to further clarify the relevance of these biochemical observations to the viral induced transformation of cells and to correlate various enzyme activity changes with other cell membrane biochemical changes (glycoproteins, virus induced antigens as detectable by immune cytolysis technique, etc.). We also plan to relate these changes, and the changes caused by the amino-sugar biosynthesis modifiers, to changes in cell immunogenicity and in tumorigenicity in syngeneic and allogeneic mouse systems.

Honors and Awards: None

Publications:

Mora, P. T., and Brady, R. O.: Changes in cell membrane biochemistry upon transformation in culture by tumorigenic DNA viruses. Transplantation Proceedings 3: 1213-1215, 1971.

Mora, P. T., Cumar, F. A., and Brady, R. O.: A common biochemical change in SV40 and polyoma virus transformed mouse cells coupled to control of cell growth in culture. Virology 46: 60-72, 1971.

Dijong, I., Mora, P. T., and Brady, R. O.: Gas chromatographic determination of gangliosides in mouse cell lines and in virally transformed derivative lines. Biochemistry 10: 4039-4044, 1971.

Brady, R. O., Mora, P. T., and Fishman, P. T.: Membrane components and enzymes in virally transformed cells. FASEB Conference on Membranes in Growth, Differentiation and Neoplasia, Federation Proceedings, in press.

Serial No. NCI-5531
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Nature and aggregation of subunits of enzymes.

Previous Serial Number: Same

Principal Investigator: Samuel W. Luborsky

Other Investigators: Sidney Shifrin

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: To determine the properties of L-asparaginase by a variety of physical and chemical techniques and to study the relationship between structure and enzyme activity.

Methods Employed: Analytical ultracentrifugation:sedimentation velocity was used to monitor enzyme dissociation; polyacrylamide disc gel electrophoresis; dialysis; spectrophotometry; enzyme activity.

Major Findings: The dissociation and inactivation of L-asparaginase by Mg^{+2} was studied. Magnesium acetate was found to be an easier reagent to work with than $MgCl_2$. A suitable assay system was unsuccessfully sought for enzyme activity in the presence of Mg^{+2}. Nesslerization, hydroxamate formation, or electrophoretic separation of ^{14}C-aspartic acid from assay mixtures containing ^{14}C-asparagine were all unsuccessfully tried. Sedimentation velocity experiments indicated that dissociation is complete at a concentration of 2M Mg^{+2}. This effect is completely reversible when Mg^{+2} is dialyzed away.

Preliminary studies were undertaken to determine the partial specific volume ($\bar{v}$) of the enzyme and of its dissociated subunits

by the technique proposed by Schachman. Precise values of $\bar{v}$ are required for the calculation of molecular weights from sedimentation equilibrium studies. The $\bar{v}$ can be obtained from the difference in slope at sedimentation equilibrium of the concentration distribution of the enzyme in water and in D_2O solutions. Densities of buffer solutions in water and in D_2O were measured. With these values and the observed sedimentation equilibrium distributions, a $\bar{v}$ of 0.74$_1$ was obtained. The currently accepted value is 0.73, an estimate based on the protein amino acid composition.

Studies were carried out on the effect of heat on the enzymatic activity and molecular structure of L-asparaginase. Enzyme activity decayed exponentially with time of boiling, and was more labile at lower protein concentrations. Activity decayed with first-order rate constants of 0.100 and 0.130 $min.^{-1}$ for protein concentrations of 4 and 0.07 mg/ml respectively. Concomitant with this loss in activity was the dissociation to monomer of the native 7s tetramer. The percent activity remaining after 20 minutes of boiling showed a rough correlation with the percent of 7s tetramer remaining. The difference spectra (heated vs unheated) were consistent with the conclusion that the enzyme had been denatured by the heat treatment. These spectra were similar to those obtained upon denaturation of other proteins, with difference bands appearing at 282, 288, and 293 nm, reflecting changes in the environments of tyrosine, tyrosine and tryptophan, and tryptophan residues respectively.

Significance to Biomedical Research and the Program of the Institute: The degree of aggregation of an enzyme is often the basis of a cellular control mechanism of enzyme activity. Studies of the stability of such interactions are thus important in attempting to understand such mechanisms and the nature of the forces responsible for the stability of the complex. Moreover, since L-asparaginase possesses significant anti-lymphoma activity, an understanding of its properties may help one to enhance this activity or to diminish some of its undesirable side-effects.

Proposed Course of the Project: The dissociation induced by Mg^{++} will be studied further to determine the nature of binding site(s) and mechanism of the dissociation. Physical characterization will continue with further experiments to determine $\bar{v}$ and molecular weight for the native enzyme as well as for the variously produced subunits. The fluorescence emission spectra of Mg-treated and of boiled enzyme will be examined.

Honors and Awards: None

Publications:

Shifrin, S., Luborsky, S. W., and Grochowski, B. J.: L-asparaginase from Escherichia coli B: Physicochemical studies of the dissociation process. J. Biol. Chem. 246: 7708-7714, 1971.

Serial No. NCI-5538
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Chemical modification of tyrosyl residues of asparaginase.

Previous Serial Number: None

Principal Investigator: Sidney Shifrin

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 2
Professional: 1
Other: 1

Project Description:

Objectives: Attempts to modify the tyrosyl residues of L-asparaginase from E. coli B were made using several different reagents including tetranitromethane, iodine and acetylimidazole. The chemical reactions with the enzyme were carried out in buffer solution and in 8M urea. The reactions in buffer were carried out to determine if tyrosyl residues that are accessible to the reagent are involved in the catalytic site and whether the modified enzyme can be reconstituted from urea. The enzyme in urea presumably makes all tyrosyl residues available for reaction including those that may be involved in subunit interactions.

Methods Employed: The enzyme was reacted with tetranitromethane, iodine in potassium iodide or N-acetylimidazole. The physicochemical properties that were studied included sedimentation velocity ultracentrifugation, disc gel electrophoresis, absorption spectroscopy, fluorescence spectroscopy, circular dichroism at the same time that the enzyme activity was followed.

Major Findings: At low molar ratios of tetranitromethane, 60% of the L-asparaginase aggregates to high molecular weight polymers without nitrating any tyrosyl residues as determined

from the absorbance at 428 nm. As the ratio of tetranitromethane to enzyme increases, there is more extensive aggregation and these aggregates contain nitrotyrosyl groups.

The large molecular weight species were separated from the tetramer on Sephadex G-100 columns and their catalytic and physicochemical properties were determined. The tetramer has the normal specific activity of native asparaginase while aggregation reduces the activity of the enzyme. Both tetramer and aggregates has the same number of normal and abnormal tyrosines suggesting that cross-linking in these large molecular weight species does not take place through tyrosyl residues.

The crosslinking in these aggregates is not so extensive that the tryptophan and tyrosyl environments cannot be perturbed by urea or sodium dodecyl sulfate as measured by ultraviolet difference spectroscopy and fluorescence.

Since nitration was hampered by this undesirable side reaction, we attempted iodination as a modifying reagent. As in previous studies we employed the reagent over a broad range of molar ratios. When 9.5 tyrosyls have been modified to the monoiodo- and di-iodo derivatives, all catalytic activity is lost. However, the tetrameric structure is maintained in these derivatives. Fluorescence spectroscopy shows that the emission at 317 nm is quenched which probably results when energy is transferred from the tryptophan to the iodo-tyrosine residues.

Iodination of asparaginase in 8M urea prevents the subunits from reaggregating to the active or inactive tetramer. While these results initially suggest that tyrosyl residues may be involved in subunit interactions, iodine may react with amino side chains other than tyrosine and because of its bulk might introduce many irreversible changes in conformation. We therefore have examined the reaction of N-acetylimidazole with L-asparaginase.

N-Acetylimidazole offers the distinct advantage not found with the other tyrosine modifying groups of being reversible. In addition it would interfere with hydrogen bonding more directly since it forms O-acetyltyrosine. At low molar ratios of acetylimidazole between 5 and 7 tyrosyl residues can be acetylated without effecting enzyme activity. At somewhat higher ratios of the reagent an additional 8 to 10 tyrosines can be acetylated with a resultant loss of 70% of the catalytic activity. The activity can be completely recovered by removing the acetyl group with hydroxylamine.

Acetylation of these tyrosyl residues does not cause the enzyme to dissociate nor does it interfere with the reassociation of subunits. This may be due to the participation of the

important tyrosyl residues in intersubunit interactions. Therefore, the enzyme was first dissociated in 8M urea to expose all tyrosyl residues to the reagent. All 44 tyrosyl residues could be acetylated which interfered with the reassociation of the subunits. Removal of these groups with hydroxylamine did not, however, allow reconstitution of the tetramer. Ultraviolet difference spectroscopy and fluorescence studies indicated that acetylimidazole produced some irreversible changes in urea that was responsible for the failure to reassociate.

Although the role of tyrosine in subunit interactions is still undefined, the results indicate that 5 or 7 tyrosines which are most reactive are not involved in the catalytic site. The next 8 to 10 tyrosines that are somewhat less reactive are partially involved in the active site and none of the tyrosines that are accessible to acetylimidazole are involved in subunit interactions.

Significance to Biomedical Research and the Program of the Institute: An understanding of the nature of the active site of L-asparaginase or the types of forces through which subunits are associated would assist in the preparation of derivatives of the enzyme that would enhance the activity and perhaps allow the enzyme to retain its tetrameric structure for prolonged periods so that it will be more useful in the treatment of leukemia.

Proposed Course of the Project: Further studies through which labeled compounds may be added to the tyrosyl groups such as spin-labeled derivatives are being planned. These reporter groups will allow studies to be made of enzyme-substrate interactions.

Honors and Awards: None

Publications:

Shifrin, S., and Solis, B. G.: L-Asparaginase from Escherichia coli B. Chemical modification of tyrosyl residues. J. Biol. Chem. 247: July, 1972.

Shifrin, S., and Solis, B. G.: Reaction of N-acetylimidazole with L-asparaginase. Molecular Pharmacology (submitted for publication).

Serial No. NCI-5539
1. Lab. of Cell Biology
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Succinylation and subunit interactions of L-asparaginase.

Previous Serial Number: None

Principal Investigator: Sidney Shifrin

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: L-Asparaginase from Escherichia coli B was succinylated with ^{14}C-succinic anhydride over a wide range of molar ratios of the reagent. The purpose of the experiment was to study the effect of succinylation on the catalytic activity of the enzyme, on the degree of dissociation into subunits, and on other physicochemical parameters.

Methods Employed: The physicochemical parameters employed in this study included sedimentation velocity, absorption and fluorescence spectroscopy, polyacrylamide disc gel electrophoresis and radioactive labeling methods.

Major Findings: There are 84 lysyl groups in a tetramer of asparaginase which are potentially available for reaction with succinic anhydride. When the ratio of succinic anhydride to lysine was 1:4, 15 positively charged lysyl residues were succinylated to form the negatively charged succinamide. The enzyme activity was increased and the tetrameric structure was retained. A somewhat larger ratio of anhydride:lysyl residue (1:2) succinylated 22 lysyl residues without effecting the state of aggregation while enhancing the activity 25%. The most apparent change in properties of this

derivative compared to the native enzyme is its migration on gels in an electric field since 22 of the positively charged lysines have been replaced by negative charges. This property will be extremely valuable in the formation of hybrids which will be discussed soon. When succinic anhydride is added to the enzyme in a ratio of 1 succinic anhydride per lysine, 35 lysyl groups (40%) become succinylated and again full activity is retained as is the tetramer. Succinylation of more than 35 of the 84 lysyl residues causes destabilization of the tetramer probably because of electrostatic repulsions and succinylated subunits are produced. At the highest concentration of succinic anhydride used in these studies (19 anhydride/lysine) more than 84 succinyl residues are bound to the enzyme. We suggest that the succinic anhydride also reacts with serine and threonine residues. At ratios of 8:1, the tetramer is completely dissociated to the monomeric subunits.

There was a direct correlation between the enzyme activity and the amount of tetramer present. By separating the succinylated tetramer from the succinylated subunit on sucrose gradients we found that the tetramer did not contain more than 35 succinyl groups and was fully active while the subunit contained increasing larger amounts of the radio-label and was, of course, inactive.

Difference ultraviolet absorption spectroscopy, fluorescence spectroscopy and circular dichroic spectra indicate that the succinylated subunit has lost all of its helical structure.

The succinylated tetramers (1:4, 1:2 or 1:1) provide excellent starting material for the formation of hybrids with unmodified subunits of asparaginase. In order to qualify as a proper derivative the modified enzyme must meet certain criteria.

a) The chemical reaction must produce a homogeneous product. This criterion was satisfied by all three samples.

b) The derivative must have a different electrophoretic mobility from the native enzyme. This criterion was satisfied by the 1:2 and 1:1 samples only since the 1:4 was so sparsely labeled that its electrophoretic mobility remained unchanged.

c) The intersubunit forces must not be much greater or much less than the forces in the native enzyme. Again both 1:2 and 1:1 samples meet this requirement. And

d) The dissociated modified subunit can be reconstituted to the aggregate. Only the 1:2 samples satisfies this requirement. The 1:1 sample cannot reassociate. When we mixed the 1:2 succinyl asparaginase with the unmodified enzyme in 8M urea and renatured by removal of urea, the

sample when applied to polyacrylamide disc gels showed 5 bands which were assigned as follows:

(1) the most rapidly migrating band represented four succinylated subunits (S_4);

(2) the next slower band contains 3 succinyl subunits and 1 native one (S_3N_1);

(3) S_2N_2

(4) S_1N_3, and

(5) N_4

According to these criteria, asparaginase and succinyl asparaginase are each tetramers with identical subunits.

All of the tetrameric succinylated samples were tested for their antileukemic activity through the help of Dr. John Venditti and were found to be equally as active as the native unmodified enzyme.

Significance to Biomedical Research and the Program of the Institute: L-Asparaginase has been found to be useful in the treatment of acute lymphocytic leukemia yet little is known about the chemical nature of its catalytically active site or about the forces that hold the subunits together into the active tetramer. The studies described in this report have indicated that lysyl residues are not involved in the catalytically active site but do participate in subunit interactions probably through electrostatic forces. These studies also demonstrated that all four subunits are identical.

Proposed Course of the Project: Further chemical studies are being carried out with asparaginase to establish the active site and the nature of intersubunit forces.

Honors and Awards: None

Publications:

Shifrin, S., Luborsky, S. W., and Grochowski, B. J.: L-Asparaginase from Escherichia coli B. Physicochemical studies of the dissociation process. J. Biol. Chem. 246: 7708, 1971.

Shifrin, S., and Grochowski, B. J.: L-Asparaginase from Escherichia coli B. Succinylation and subunit interactions J. Biol. Chem. 247: 1048, 1972.

Serial No. NCI-5550
1. Laboratory of Cell Biology
2. Viral Oncogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Oncogenic RNA Viruses

Previous Serial Number: Same

Principal Investigator: Kenneth S. S. Chang, M.D., Sc.D.

Other Investigator: Dr. L. W. Law (C:LCB-3200)

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

1. Isolation of oncogenic RNA virus from SJL/J strain of mouse.

In order to understand the natural history of a murine leukemia virus (MuLV) in SJL/J mice and to investigate the etiological relationship of the virus to the Hodgkin's like reticulum cell neoplasm found spontaneously in high incidence in this strain of mouse, an attempt was made to isolate MuLV from spleens of SJL/J mice of various ages. The virus was found to be N-tropic, antigenically related to AKR-Gross virus, and present in spleens in titers ranging from 10^1 to 10^2. The isolation rate was highest in ages 4 and 8 months, and the titers of naturally occurring neutralizing antibody was found to be higher in these age groups. The virus was used as a helper for rescue of MSV from nonproducer cells and the resultant MSV(SJL) produced a subcutaneous "atypical granuloma" in thymectomized and antilymphocyte serum-treated SJL/J mice. It also produced a lymphoblastic leukemia 5 months after neonatal injection. Immunogenicity of these tumors and viruses in SJL/J mice in terms of cytotoxic antibody and neutralizing antibody production, and protection against spontaneous leukemia and reticulum cell neoplasms is being studied.

2. Studies on the role of oncogenic RNA virus in human Hodgkin's disease.

By uridine-^{3}H labeling technique, an RNA virus sedimenting at a density of 1.20 gm/ml in sucrose density gradient centrifugation was detected in the supernatant fluid of a culture of lymph node biopsied from a patient with Hodgkin's disease. Efforts have been made to examine its infectivity, MSV genome rescue activity, and immunologic reactivity. Preliminary evidence indicates that the virus can act as a helper for MSV genome present in a nonproducer cell line. A similar culture from another patient was negative. A third culture is being attempted. It is hoped that findings and techniques gained in SJL/J mouse study may serve as a model for the above investigation.

It is planned to collect more cultures of lymph nodes from Hodgkin's and other disease patients. Serum samples shall be collected and their immunologic reactions against this virus be examined.

3. Interaction of visna virus with MuLV.

In order to define the importance of slow virus infection in neoplastic diseases, visna virus, which is a member of this group and related to RNA oncogenic viruses, was studied for its interaction with the MSV and MuLV. Focus-forming and replicating activities of MSV and MuLV in normal rat kidney (NRK) cells and in NIH Swiss mouse embryo (ME) cells were interfered by pre-treatment of these cells with visna virus. Effects of UV-irradiation of visna virus on its infectivity, interfering activity and cell-transforming activity are being investigated. Further study is planned to elucidate the mechanism of interference and cell transformation by visna virus.

4. Studies on the MuLV in NZB mice.

Attempts have been made to isolate the MuLV present in NZB mice, which are noted for their immunologic abnormalities and high incidence of reticulum cell neoplasm. Although the restricted host specificity of this virus presents difficulty in isolation from the organs of NZB mice, it was possible to isolate it from NZB embryo cultures in the form of MSV(NZB) by MSV genome rescue procedure. Among various types of cells tested, NRK cell line was the only type of cell that transformed after MSV(NZB) infection. Further, it was possible to use this focus assay for demonstration of the presence of neutralizing antibody in the serum of NZB mice of various ages. Natural occurrence of viral antibody in NZB mice has been demonstrated by immuno-fluorescence and immunoelectron microscopic techniques, but neutralizing activity of the antibody has not been demonstrated previously. The role of MuLV and its antibody in pathogenesis of NZB disease will be further investigated.

5. Studies on MSV(RaLV).

In vitro adaptation of MSV(RaLV) - formerly designated MSV (O) - to mouse embryo cells has resulted in a few clones which continued to produce virus particles detectable by uridine-^{3}H labeling technique. Although attempts at rescue of MSV genome from these cells failed, other methods of activation are being tried. *In vivo* passage of UV-irradiated MSB_1 cells in BALB/c mice has been carried out for more than 30 generations. The tumor was invariably transplantable, but regression followed. Since a purified, concentrated preparation of MSV(RaLV) became available, oncogenicity of this virus in mice and rats is being investigated. The presence of reverse transcriptase in this virus preparation was asserted but the activity was rather low compared with a similar preparation of Rauscher leukemia virus. In complement fixation

tests, the virus reacted with a broad reacting, rat anti-MSV(MLV) serum only after treatment with ether. The antigenic specificities of the envelope and gs antigen are being investigated.

Publications:

Chang, S. S., Gilden, R.V., and Huebner, R. J.: Rescue of murine sarcoma virus genome in mixed cultures of non-producer hamster tumour cells and helper virus carrier cell lines. J. Gen. Virol. 10: 107-110, 1971.

Law, L. W., and Chang, S.S.: Effects of antilymphocyte serum (ALS) on the induction of lymphocytic leukemia in mice. Proc. Soc. Exp. Biol. Med. 136: 420-425, 1971.

Serial No. NCI-4833
1. Laboratory of Cell Biology
2. Ultrastructural Biology Sect.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Electron microscope and biological studies of normal, malignant and virus infected cells and cell fractions

Previous Serial Number: Same

Principal Investigator: Dr. Nelson A. Wivel

Other Investigators: Dr. Philip M. Grimley (NCI-861)
Dr. E. Kuff (NCI-366)
Dr. G. Smith (NCI-3526)

Man Years: Total - 2
Professional - 1
Other - 1

Project Description:

Objectives:

The Ultrastructural Biology Section is concerned with the study of various factors which influence cell differentiation and malignant transformation. Particular emphasis is given to systems in which viruses represent the carcinogenic agent. The projects undertaken require the correlation of data from various disciplines such as electron microscopy, virology, immunology and biochemistry.

Methods Employed:

The principal methods involve the visualization of appropriate selected biological materials in the electron microscope, the use of tissue culture for propagation of viruses, the use of differential ultracentrifugation to isolate cell and virus fractions, and the detection of viral antigens by a variety of immunological techniques.

Major Findings:

1. A collaborative effort with Dr. P. Grimley (NCI-861), has involved the study of human thyroid medullary carcinoma. This particular type of tumor is a histologically distinct neoplasm, it is probably derived from calcitonin-secreting parafollicular cells, it is often hormonally active,

and the susceptibility to neoplasia may be inherited as an autosomal dominant trait. Two separate tumors were used to establish tissue culture lines. These lines were assayed for alkaline phosphatase activity since the putative cell of origin has been shown to have this enzyme; they were found to be consistently positive. In order to prolong the in vitro life span of these endocrine cells, they were transformed with SV40. Following transformation the alkaline phosphatase levels were markedly depressed or undetectable. When hydrocortisone was added to the tissue culture system, the enzyme activity returned to preinfection levels. This increase in alkaline phosphatase was dependent on the continuous presence of steroid; when steroid was withdrawn, there was a loss of activity to pretreatment levels. No increase in enzyme levels was detected in uninfected tumor cells treated with hydrocortisone. All of the data presently available from these two tissue culture lines are consistent with the hypothesis that SV40 may repress a cellular gene. Since SV40 transformed human tumor cells have a finite period of survival, present efforts are directed toward the identification of a more stable mammalian cell line which contains alkaline phosphatase and which can be permanently transformed by SV40. As the experimental system is constituted it offers the opportunity to study interactions between cell and viral genes and thus one can measure additional parameters of malignant transformation.

2. In a continuing investigation of intracisternal A-particles, virus-like particles which are found in a variety of normal and neoplastic mouse tissues, certain distinctive characteristics have been established. This work is being done in collaboration with Dr. E. Kuff (NCI-366). Particles isolated from several different mouse myeloma lines and from cultured neuroblastoma (C1300) cells all contain a major structural protein with an apparent molecular weight of 70,000 as estimated by electrophoretic mobility in SDS-containing polyacrylamide gels, The size distribution of A particle proteins is different from that observed for extracellular murine leukemia and mammary tumor viruses. Rabbit antiserum has been developed that reacts with the major structural protein in both C-F and immunodiffusion assays. The antigen can be detected in A-particle isolates from tumors, but not in preparations from normal mouse organs or samples of leukemia and mammary tumor virus. Conversely, isolated A-particles do not react in C-F or immunodiffusion assays with antisera against leukemia virus antigens. Recently an extract from 30 grams of BALB/c mouse embryo tissues was shown to contain antigen and particles as seen by electron microscopy. This finding plus the fact that A-particles are present in both male and female gonadal tissue make it most probable that vertical transmission takes place, a feature shared in common with the leukemia viruses. Experiments have been initiated which will yield data concerning the presence of: (1) a reverse transcriptase; (2) the presence of a high molecular weight RNA of the type seen in the

known oncogenic RNA viruses; (3) the rate of synthesis and degradation of particles in both animal and tissue culture systems. It is anticipated that these results will help determine whether or not A-particles reflect the expression of a viral genome as opposed to the abnormal production of a cellular constituent.

3. A study of intracytoplasmic A-particles is being done in association with Dr. G. Smith (NCI-3526). Because of electron microscopic observations in mouse mammary tumors, it was proposed that intracytoplasmic A-particles were the precursors of the B particles (mammary tumor virus). However, since these A-particles were not isolated it wasn't possible to obtain unequivocal evidence to support this hypothesis. Recently we have developed a technique for the isolation of intracytoplasmic A particle inclusions from mouse mammary tumors and interstitial cell tumors and thus are in a position to compare them with mammary tumor virions. Preliminary evidence indicates that the gel electrophoresis patterns of A particles and B particles are distinct, suggesting that a reorientation of structural protein occurs during the maturational process, assuming that the two particles are related. BALB/c and C3H mice have been inoculated with purified intracytoplasmic A-particles but no tumors have developed over a period of approximately three months. Intracisternal and intracytoplasmic A-particles have been compared immunologically; when using specific antisera to the two types of particles there is no cross reaction in C-F assay or immunodiffusion. Present and future efforts are directed toward a more complete analysis of the intracytoplasmic A-particles from the standpoint of biological activity, biophysical properties and immunological profile.

Relationship of research to National Cancer Plan:

Project Area - 10

Approach Elements - 2

Approach - 4

Objective - 1

Serial No. NCI-4833
1. Laboratory of Cell Biology
2. Ultrastructural Biology Sect.
3. Bethesda, Md.

Publications:

Kuff, E., Lueders, K., Ozer H., and Wivel, N.: Some structural and antigenic properties of intracisternal A particles occurring in mouse tumors. Proc. Nat. Acad. Sci. 69: 218-222, 1972

Wivel, N. A., and Grimley, P. M.: Reversible suppression of alkaline phosphatase in human thyroid medullary carcinoma cells transformed by SV40. Proc. Soc. Exp. Biol. Med. 139: 627-630, 1972.

Smith, G. H., and Wivel, N. A.: Isolation and partial characterization of intracytoplasmic A-particles. Virology, 48: (April, 1972).

SUMMARY STATEMENT
LABORATORY OF MOLECULAR BIOLOGY
GL&C, NCI
July 1, 1971 - June 30, 1972

The research effort in the Laboratory of Molecular Biology is divided into three general areas.

1. How is the expression of genetic information controlled in bacterial and animal cells?

2. What is the molecular basis of genetic recombination?

3. Are some of the properties of cancer cells due to alterations in the cyclic AMP control system?

Regulation of gene expression - Of the approximately 10,000 genes of E. coli, we only understand, at a biochemical level, how the expression of a few of these is controlled. The factor that has made this understanding possible is cyclic AMP, for it is that molecule together with its specific cyclic AMP receptor protein (CRP) that enables RNA polymerase to transcribe a variety of the genes of the E. coli chromosome. Last year we reported that for the first time controlled transcription of two bacterial genes had been achieved in a cell-free system. The genes were those of the lactose (lac) and galactose (gal) operons. Cyclic AMP and CRP act to allow RNA polymerase to bind to the gene promoters in a form ready to initiate transcription. The past year has been spent in trying to understand the nature of this process.

For these studies the CRP has been purified to homogeneity and its properties studied by W. B. Anderson. It is a very basic protein (pI 9.1) composed of two identical subunits of 22,500 daltons each. Its amino acid composition has been determined and studies on its primary structure have been initiated. S. P. Nissley and M. Gallo have found that in the presence of cyclic AMP, CRP binds to DNA. Therefore it seems likely that CRP acts by altering DNA so that RNA polymerase can bind at a nearby site. A variety of cyclic AMP analogues have been tested by W. B. Anderson to see if they will stimulate the binding of CRP to DNA or transcription of the gal operon. Surprisingly only one analogue, tubericidin 3'5' cyclic phosphate was active. Apparently the structural requirements are very stringent for these reactions, although many cyclic nucleotides will compete for the binding of cyclic AMP to CRP. These studied indicate that the mechanism by which cyclic AMP acts is quite complex.

The availability of a system which will transcribe the lac operon has enabled us to determine how lac repressor works. Two models had been previously proposed. In one, lac repressor prevents binding of RNA polymerase to the lac promoter site; in the other the binding of the lac repressor and RNA polymerase are independent events. B. de Crombrugghe and B. Chen found that the latter model is correct.

Gal operon expression is also under the control of a specific repressor. We have been able to identify this molecule in crude extracts and have been able to purify it to a high degree. The gal repressor molecule specifically binds to DNA containing a normal gal operator; rather recently S. Nakanishi found that it represses cyclic AMP-CRP dependent transcription of gal DNA.

Control of gal operon expression is more complex than that of the lac operon. In the past year we have been joined by S. Adhya who previously did much of the genetic analysis of the galactose operon. Adhya has isolated a variety of new regulatory mutants and is currently characterizing these. DNA from the mutants is being prepared and we will be able to study how these mutants DNAs are transcribed. A few of these mutants are deleted for most of the genes of the gal operon. RNA transcribed from such a template is small enough to have its sequence determined. B. de Crombrugghe in collaboration with S. Weissman at Yale University have begun such studies.

We have also begun to study how some other regulatory molecules work. One of these, ppGpp, stimulates ß-galactosidase synthesis and lac transcription in cell-free extracts. The factors required for the action of ppGpp are being investigated by M. Aboud.

In addition to gene transcription being regulated by factors that operate at the initiation step, genes can be transcribed when they are fused to neighbors whose transcription has been initiated at a distant promoter. This probably occurs when oncogenic viruses transform cells and also occurs in E. coli lysogenized by the bacterial virus λ. K. Krell and M. Gottesman have been investigating how the bacterial genes for biotin synthesis are turned on when they are fused to the chromosome of λ. Apparently RNA polymerase begins transcribing at a promoter in the λ region and reads through into the biotin genes if conditions are favorable. Some mutations that regulate this process are under study.

Molecular Basis of Genetic Recombination - A unique feature of the bacteriophage λ is its ability to integrate into and excise from the bacterial chromosome. To understand this process, mutants defective in the regions of the λ chromosome that combine with the bacterial chromosome are needed. M. Shulman has isolated such mutants and is investigating their effects on the recombination process. His experiments indicate that excision and integration proceed by generating complementary single stranded regions at each attachment site.

Another approach to understanding the nature of the recombination process has been begun by S. Gottesman. Using DNA extracted from a special phage that contains DNA with both the bacterial and λ attachment sites, she will attempt to identify the enzymes promoting the recombination process.

Cancer and cyclic AMP - Cancer is a disease in which many cell processes go awry at once. In E. coli the inability to make cyclic AMP leads to a variety of cellular defects. Thus, a plausible explanation of how a single genetic event might lead to a variety of cellular changes would be its inability to make cyclic AMP. Last year we summarized studies indicating that

cyclic AMP might be needed for fibroblasts to behave normally, since treatment of abnormally behaving transformed fibroblasts with the cyclic AMP analogue, dibutyryl cyclic AMP (dbc), reverted some of their properties towards normal. The past year has been spent in defining which characteristics of fibroblasts are under cyclic AMP control and how transformation affects cyclic AMP metabolism.

Transformed fibroblasts are often discernable from normal cells by their altered appearance, increased growth rate, decreased adhesiveness to their substratum, altered motility and inability to synthesize some special macromolecules. Following treatment of SV40 transformed cells or L cells with dbc, G. Johnson has found that their shape becomes more elongated and flattened, their motility and growth rate slows and their adhesiveness increases. Also the synthesis of sulfated mucopolysaccharies is increased. In cells in which prostaglandin E1 raises cyclic AMP levels, similiar responses are observed.

If cyclic AMP levels in cells regulate growth rate, a direct correlation between the level of cyclic AMP and the doubling time should occur. J. Otten measured cyclic AMP levels in growing cells and observed such a relationship. He also found that cyclic AMP levels rose in contact-inhibited cells suggesting that cyclic AMP is the signal for growth arrest in such cells.

In order to determine which metabolic step controls cyclic AMP levels, we have been measuring adenylate cyclase and cyclic AMP phosphodiesterase. Surprisingly M. D'Armiento observed that the phosphodiesterase activity was high in cells with high cyclic AMP levels. This finding lead to experiments indicating that cyclic AMP induces synthesis of the diesterase, probably as part of a feedback control mechanism regulating cyclic AMP levels.

More recently in a collaborative study with J. Bader of the Chemistry Branch, NCI, we have obtained evidence that expression of a gene in the transformation region of the Rous Sarcoma Virus genome somehow lowers cyclic AMP levels. Work to elucidate the mechanism of this exciting finding is in progress.

Serial No. NCI-8000
1. Lab. of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Regulation of Gene Expression in E. coli by cyclic AMP.

Previous Serial Number: SAME

Principal Investigator: Ira H. Pastan

Other Investigators: Benoit de Crombrugghe, Wayne B. Anderson, S. Peter Nissley, Shigetada Nakanishi, Mordechai Aboud and Maxwell E. Gottesman

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	6 6/10
Professional:	5 6/10
Other:	1

Project Description:

Objective: Cyclic AMP is required for the synthesis of a variety of enzymes in E. coli, most of which are inducible. Cyclic AMP acts at a transcriptional level. Our objective is to determine how cyclic AMP, cyclic AMP receptor protein (CRP) and other factors control gene activity.

Methods Employed: Studies on mRNA and enzyme synthesis have been carried out in intact cells and in cell-free extracts. Mutants which are defective in cyclic AMP metabolism have been isolated and characterized. Proteins controlling gene transcription are isolated and their mechanism of action studied.

Major Findings: We have previously determined that cyclic AMP and cyclic AMP receptor protein (CRP)are required for transcription of the lac and gal operons.

The cyclic AMP receptor (CRP) has now been purified to apparent homogeneity and further characterized. It is a basic protein with an isoelectic point of 9.12 as determined by isoelectic focusing. There are four free sulfhydryl groups per native dimer and these account for the four half-cystines observed by amino acid analysis. Studies are in progress to determine the complete amino acid sequence of the 22,500 MW monomer; the first six amino acids in the NH_2-terminal end of the protein are Val-Leu-Gly-Lys-Pro-Gln.

Several analogues of cyclic AMP have been tested for their ability to inhibit the binding of cyclic AMP to CRP and to inhibit the cyclic AMP-CRP-dependent transcription of the gal operon. These analogues which inhibit cyclic AMP binding also decrease cyclic AMP-CRP dependent transcription. CRP exhibits a marked specificity for cyclic AMP in stimulating gal mRNA synthesis. These studies indicate that the N^6-amino group, the 2'-hydroxyl group, and the cyclic phosphate group all are involved in binding to CRP and eliciting the correct conformational change required to mediate cyclic AMP-dependent gene transcription.

Rabbit antisera has been prepared against CRP to facilitate the detection of inactive CRP as well as to detect CRP-like proteins from other sources. This CRP antisera is able to bind CRP and completely inhibit CRP-cyclic AMP dependent transcription.

To understand how the cyclic AMP-CRP complex stimulates the initiation of transcription, we have been examining the interactions between the complex and either DNA or RNA polymerase. To date no interaction of CRP with RNA polymerase has been observed. However, CRP does bind to DNA in the presence of cyclic AMP. This has been demonstrated by measuring the binding of unlabelled CRP to {^{32}P}-λh80dlac or λpgal DNA. It has also been demonstrated by measuring the binding of unlabelled DNA to {^{14}C}-CRP. In both cases the binding is specifically stimulated by cyclic AMP. Nucleotides such as cyclic GMP, which fail to stimulate transcription, fail to stimulate the binding of CRP to DNA. A puzzling finding is that CRP binds as tightly to species of DNA on which it does not promote transcription, as to those on which it does. This suggests that CRP binding sites are common, but only when they are adjacent to a potential RNA polymerase binding site does CRP promote binding of RNA polymerase.

In addition to cyclic AMP and CRP, which function to stimulate the initiation of lac transcription, transcription is repressed by the specific lac repressor. Two models have been proposed to explain how lac repressor acts. Either it prevents binding of RNA polymerase to DNA or it prevents bound polymerase from moving to the site where nucleotide polymerization begins. With the use of the inducer IPTG, which removes lac repressor bound to the lac operator, and rifampicin, which inactivates RNA polymerase not bound to the lac operator, we were able to establish that the latter model was correct. The cyclic AMP-CRP dependent binding of RNA polymerase and the binding of lac repressor are independent events. However, using lac DNA with a mutation in the lac promoter (lacp^s) that increases the efficiency of lac transcription, the binding of RNA polymerase and CRP were found to be partially competitive.

Genetic experiments in whole cells have indicated that expression of the gal operon is also under the control of a specific repressor. We have detected the presence of the gal repressor in cell-free extracts by measuring its binding to {^{32}P}-λpgal DNA. The content of gal repressor in normal cells is very low. To increase its content in extracts the gal repressor gene has been integrated into the genome of the bacteriophage λ. When the λpgalR phage is induced to replicate, there is at 5-10 fold increase in gal repressor synthesis.

From such extracts the gal repressor has been purified. The repressor specifically binds to λpgal DNA and not to λpgalO^c DNA. Binding is prevented by inducers of the gal operon transcription and this inhibition is reversed by fucose. A comparison of the properties of the gal and lac repressor is in progress.

Another molecule that controls lac operon expression is the nucleotide ppGpp. ppGpp will stimulate lac transcription with a crude preparation of RNA polymerase made from a ribosomal wash but not with pure RNA polymerase. Thus a factor present in the ribosomal wash is required for the stimulation. This factor is now being purified.

The ability to make lac and gal mRNA in vitro with only purified components, permits us to begin to determine the sequence of these messages and to examine the nature of genetic punctuation. To begin these studies we have prepared a number of λpgal phages containing a variety of mutations and deletions of gal operon genes. With these we will begin to study the nature of initiation and termination signals.

Significance: In cancer cells, genes which are normally quiet become active due to some alteration in the control of gene expression. It is not yet possible to study the expression of individual genes in animal cells. Therefore our studies on the control of the transcription of lac and gal genes by cyclic AMP, cyclic AMP receptor protein, lac repressor, gal repressor and ppGpp are a basis on which to build an understanding of how gene expression is controlled in higher organisms.

By understanding how gene activity in bacteria is controlled we then will have a basis to understand the "nature and modification of precancerous state and determination of mechanisms accounting for high degrees of stability of cell functioning" in order "to prevent conversions of cells to those capable of forming cancers (i.e., block or interfere with the proximate step, or steps, involved in conversion to capable of forming cancers"). (Objective 3, Approach 1, National Cancer Program)

Proposed Course: 1. To examine the chemical mechanism by which cyclic AMP and CRP instruct RNA polymerase to initiate gene transcription.

2. To determine the sequence of select regions of lac and gal mRNA and thereby establish if the promoter or operator regions of the gene are transcribed.

3. To search for other regulatory molecules which control gene expression, and determine how they act.

4. To determine the architecture of the genetic regions where gene transcription is initiated and terminated.

Honors and Awards: Van Meter Prize, American Thyroid Association
Boxer Lectureship - Rutgers University

Publications:

Pastan, I., Perlman, R. L., de Crombrugghe, B., and Varmus, H.: Role of cyclic AMP in Induction and Differentiation. In Bethesda, Md. (Eds.): Rall, T.W., Rodbell, M., and Condliffe, P. The Role of Adenyl Cyclase and cyclic 3',5'-AMP in Biological Systems. U. S. Gov't. Printing Ofc., 1971, pp. 201-213.

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de Crombrugghe, B., Chen, B., Anderson, W., Nissley, P., Gottesman, M. Perlman, R., and Pastan, I.: Lac DNA, RNA polymerase, cyclic AMP receptor protein, cyclic AMP, lac repressor and inducer are the essential elements for controlled lac transcription. Nature - New Biology 231: 139-142, 1971.

Nissley, P., Anderson, W., Gottesman, M., Perlman, R. L., and Pastan, I.: In vitro transcription of the gal operon requires cyclic AMP and cyclic AMP receptor protein. J. Biol. Chem. 246: 4671-4678, 1971.

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Perlman, R. L. and Pastan, I.: The Role of cyclic AMP in Bacteria. (Eds.): Stadtman, E. and Horecker, N. Current Topics in Cellular Regulations. New York, N. Y., Academic Press, 1971 3, pp. 117-134.

Parks, J. S., Gottesman, M., Shimada, K., Weisberg, R. A., Perlman, R. L., and Pastan, I.: Isolation of the gal repressor. Proc. Nat. Acad. Sci. 68: 1891-1895, 1971.

Chen, B., de Crombrugghe, B., Anderson, W., Perlman, R. L., Gottesman, M., and Pastan, I.: On the Mechanism of action of lac Repressor. Nature - New Biology 233: 67-70.

Pastan, I. and Perlman, R. L.: Regulation of Gene Transcription in E. coli. (Eds.): Greengard, P., Robison, G.A., and Paoletti, R. Advances in Cyclic Nucleotide Research. New York, N. Y., Raven Press, in press, 1972.

de Crombrugghe, B., Chen, B., Anderson, W., Gottesman, M., Perlman, R. L., and Pastan, I.: Role of cyclic Adenosine 3',5'-Monophosphate and the cyclic Adenosine 3',5'-Monophosphate Receptor Protein in the Initiation of lac Transcription. J. Biol. Chem. 246: 7343-7348, 1971.

Pastan, I.: The 1971 Nobel Prize for Medicine or Physiology (Earl Sutherland, M.D., recipient). Science 174: 392-393, 1971.

Anderson, W. B., Perlman, R. L., and Pastan, I.: Effect of Adenosine 3',5'-Monophosphate Analogues on the Activity of the cyclic Adenosine 3',5'-Monophosphate Receptor in Escherichia coli. J. Biol. Chem., in press, 1971.

Nissley, P., Anderson, W., Gallo, M., Perlman, R. L., and Pastan, I.: The Binding of cyclic AMP Receptor to DNA. J. Biol. Chem., in press, 1972.

Pastan, I. and de Crombrugghe, B.: Control of Expression of Genetic Information in E. coli by cyclic AMP. In Stockholm, Sweden. Vth Karolinska Symposia. (Ed.): Diczfalusy, E., in press, 1972.

Serial No. NCI-8001
1. Lab. of Molecular Biology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Role of cyclic AMP in the Regulation of Growth and Morphology of Transformed and Normal Fibroblasts

Previous Serial Number: SAME

Principal Investigators: Ira H. Pastan and George S. Johnson

Other Investigators: Mark Willingham, Jacques Otten, Massimo D'Armiento, Charles Peery, David Oyer, John F. Goggins and John Bader

Cooperating Units: Experimental Pathology Branch, National Institute of Dental Research and Chemistry Branch, National Cancer Institute

Man Years (July 1, 1971 through June 30, 1972)

Total:	7 7/10
Professional:	6 7/10
Other:	1

Project Description:

Objective: To investigate how cyclic AMP regulates the growth and morphology of normal and transformed fibroblasts, and to determine the nature of the alteration of cyclic AMP metabolism in transformed fibroblasts.

Methods Employed: Cell culture and standard biochemical analyses of the enzymes involved in cyclic AMP metabolism and the synthesis of macromolecules.

Major Findings: Transformed fibroblasts grown in tissue culture have a number of characteristics that differentiate them from normal cells. These frequently include a change in morphology, a decreased adherence to substratum, an increase in growth rate, a loss of contact inhibition of growth and movement and a decreased ability to synthesize specialized macromolecules such as sulfated mucopolysaccharides. When such cells are treated with dibutyryl cyclic AMP (DBC) and substances that inhibit cyclic AMP phosphodiesterases such as theophylline, many of these characteristics return towards normal.

Morphology: Cells treated with DBC and theophylline become flattened and elongated. With normal fibroblasts the change is small, since they are already in this configuration. However, with some transformed cells the effects are

quite striking. We have observed varying degrees of response with a variety of clones derived from 3T3 cells transformed by SV40, polyoma virus and murine sarcoma virus, as well as with human osteosarcoma cells.

Adhesiveness: We have quantitated the increase in adhesiveness by measuring the rate at which cells are removed from their substratum by trypsin or EGTA. DBC treated cells show a maximum increase in adhesiveness after only 30 min of treatment, and the process is reversed 30 min after DBC removal. Increased adhesiveness is not blocked by inhibitors of protein synthesis indicating that an effect of DBC on protein synthesis is not required for the response.

Motility: The addition of DBC to L-929 cells also decreases their motility. The phenomenon, like the change in adhesiveness, occurs rapidly and is rapidly reversed.

Growth: DBC decreases the growth rate of all transformed lines studied. With L-929 cells, one line of SV40-3T3 cells and 3T3-Pyll cells, no change in saturation density was observed. However both the growth rate and saturation density of 3T3 cells are decreased.

Macromolecules: 3T3 SV40 clone X has a greatly reduced rate of sulfated mucopolysaccharide production. In this line mucopolysaccharide accumulation is stimulated by DBC.

Cyclic AMP Phosphodiesterase: Unexpectedly 3T3 cells with high cyclic AMP levels were found to have elevated levels of cAMP phosphodiesterase. This finding suggests that cAMP is an inducer of cAMP phosphodiesterase. When 3T3 SV40 transformed cells with low cyclic AMP levels were treated with DBC, production of the enzyme was induced.

Cyclic AMP Levels: The levels of cyclic AMP were measured in a variety of cell lines with different growth rates. There was a striking inverse correlation between the growth rate and the cyclic AMP level. Insulin, serum, and trypsin which stimulate growth, lower cyclic AMP levels, whereas PGE1 which raises cyclic AMP levels decreases growth rates. These findings are consistent with the hypothesis that cyclic AMP regulates growth rates. Further cyclic AMP rose in confluent contact inhibited 3T3 cells, whereas it fell in non-contact inhibited cells. This finding suggests that a rise in cyclic AMP levels is the signal for confluent 3T3 cells to cease growing.

When a temperature sensitive mutant of Rous Sarcoma Virus {(RSV-TA) isolated by John Bader} is grown in cells at 41°, the cells appear normal and the cyclic AMP levels are high. When the cells are shifted to 36°, cyclic AMP levels fall before major evidence of morphologic transformation. Further, treatment of these cells with DBC and theophylline prevents the appearance of the transformed phenotype.

Significance: Part of the reason for the abnormal behavior of transformed cells in culture is their inability to maintain normal cyclic AMP levels. If one could raise levels in these cells, one might control the growth of certain types of cancers.

These studies on the "nature and modification of precancerous state and determination of mechanisms accounting for high degrees of stability of cell functioning" will serve "to prevent conversions of cells to those capable of forming cancers (i.e., block or interfere with the proximate step, or steps, involved in conversion to capable of forming cancers)". (Objective 3, Approach 2, National Cancer Program)

Proposed Course: We will examine how the transformation event alters the ability of cells to maintain normal cyclic AMP levels. We also will study how cyclic AMP works to slow growth and produce its other effects. We will investigate why some cells are much less responsive to cyclic AMP than others.

Honors and Awards: None

Publications:

Johnson, G. S., Friedman, R. M., and Pastan, I.: Cyclic AMP treated sarcoma cells acquire several morphological characteristics of normal fibroblasts. Ann. N. Y. Acad. Sci. 185: 413-416, 1971.

Otten, J., Johnson, G., and Pastan, I.: Cyclic AMP levels in Fibroblasts: Relationship to Growth Rate and Contact Inhibition of Growth. Biochem. Biophys. Res. Commun. 44: 1192-1198, 1971.

Johnson, G. S. and Pastan, I.: Change in Growth and Morphology of Fibroblasts by Prostaglandins. J. Nat. Cancer Inst. 47: 1357-1364, 1971.

Johnson, G. S., Morgan, W. D., and Pastan, I.: Regulation of Cell Motility by cyclic AMP. Nature - New Biology 235: 54-56, 1972.

Pastan, Ira H.: The Mechanism of Action of Protein and Polypeptide Hormones. In Wash., D. C. (Eds.) F. W. Sunderman, M. D. and F. W. Sunderman, Jr., M. D. Laboratory Diagnosis of Endocrine Diseases. St. Louis, Missouri, Warren H. Green, Inc. pp. 111-113, 1971.

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Johnson, G. S., Pastan, I., Peery, C. V., and Otten, J.: The Role of Prostaglandins in the Regulation of Growth and Morphology of Transformed Fibroblasts. In Carmel, Calif. Prostaglandins in Cellular Biology. New York, N. Y., Plenum Publishing Co., 1972, pp. 195-219.

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D'Armiento, M., Johnson, G. S., and Pastan, I.: Regulation of Adenosine 3',5'-Cyclic Monophosphate Phosphodiesterase Activity in Fibroblasts by Intracellular Concentrations of Cyclic Adenosine Monophosphate. Proc. Nat. Acad. Sci., USA 69: 459-462, 1972.

Johnson, G. S. and Pastan, I.: Cyclic AMP regulates the Adhesion of Fibroblasts to Substratum. Nature - New Biology, in press, 1972.

Pastan, Ira H.: Current Directions in Research on cyclic AMP. In Bethesda, Md. (Eds.): Anfinsen, C. B., Goldberger, R. F., and Schechter, A. N. Current Topics in Biochemistry. New York, N. Y. Academic Press, Inc., in press, 1972.

Johnson, G. S. and Pastan, I.: $N^6,O^{2'}$-Dibutyryl Adenosine 3',5'-Monophosphate Induces Pigment Production in Melanoma Cells. Nature, in press, 1972.

Otten, J., Bader, J., Johnson, G. S., and Pastan, I.: A Mutation in a Rous Sarcoma Virus Gene that controls Adenosine 3',5'-Monophosphate Levels and Transformation. J. Biol. Chem. 247: 1632-33, 1972.

Serial No. NCI-8003
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Integration and Excision of Phage λ.

Previous Serial Number: SAME

Principal Investigators: Marc Shulman and Maxwell E. Gottesman

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	1 3/10
Professional:	1 3/10
Other:	

Project Description:

Objectives: To study the mechanisms involved in integration and excision by phage λ.

Methods Employed: Standard microbiological techniques and published modifications developed in this laboratory have been employed.

Major Findings: We have begun to characterize the λ attachment sites, the regions of DNA which are the substrate of integration and excision of phage λ. To study these regions we are isolating phage which have point mutations in the attachment sites. To isolate these mutants we employ an unusual transducing phage which carries the two hybrid attachment sites and is thus itself a substrate for intramolecular recombination promoted by the site-specific phage excision system.

Among the products of excision by the mutant phage are phage which are heterozygous for the mutation. This result suggests that excision and integration proceed by generating complementary single stranded regions from each attachment site and that the mutation lies in this special region. The mutation is not associated with a unique attachment site, i.e., after integration or excision it can appear in phage, bacterial and either prophage attachment site. This segregation pattern of the mutation is qualitatively consistent with the conclusion that it lies in a region used to generate complementary single strands.

Significance to Bio-medical Research and the Program of the Institute:

These studies elucidate the mechanisms of phage integration and excision. These mechanisms may be of importance in a variety of virus-host systems, especially in regard to those viruses that cause human cancer.

It provides information of potential value in determing the nature of oncogenic cell transformation and thus may ultimately permit the prevention of conversion of normal to tumorogenic cells. (Objective 3, Approach 2, National Cancer Program)

Proposed Course: We will attempt to characterize the mutations by following the course of radioactively labeled phage DNA after infection. In particular, we hope to isolate intermediates in the reaction as labeled DNA fragments, accumulating as a consequence of the att mutation.

Honors and Awards: None

Publications:

Shulman, Marc and Gottesman, Maxwell E.: λatt^2: A Transducing Phage capable of Intramolecular Int-Xis Promoted Recombination. In Cold Spring Harbor, N. Y. (Ed.): Hershey, A. D. Bacteriophage Lambda. N.Y., N.Y., Cold Spring Harbor Laboratory, 1971, pp. 477-487.

Serial No. NCI-8004
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Control by lambda genes of the expression of bacterial genes.

Previous Serial Number: SAME

Principal Investigators: Kenneth Krell and Maxwell E. Gottesman

Other Investigators: John S. Parks

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	1 3/10
Professional:	1 3/10
Other:	

Project Description:

Objectives: To characterize the mechanisms by which lambda genes control the expression of bacterial genes, specifically those of the biotin operon.

Methods Employed: Standard microbiological and biochemical techniques as well as those developed by the principal investigator and in this laboratory.

Major Findings: The induction of E. coli K-12 λ lysogens gives rise to the constitutive synthesis of desthiobiotin synthetase (DTBS), and 7-keto-8-amino pelargonic acid aminotransferase (KAPS), collectively termed "bio escape synthesis", but only when the prophage carries a deletion in the b2 region. The genetic determinant of DTBS is the bioD gene, the rightmost cistron of the bio operon. The bioA gene, which codes for KAPS, maps at the left end of the operon. DTBS escape synthesis is shown to be primarily due to transcription initiated at a promoter activated by phage gene Q product. Neither prophage replication nor induction are essential; DTBS escape synthesis can also be demonstrated in repressed λb2 lysogens after infection with a heteroimmune phage. The escape synthesis occurs whether the superinfecting phage is $b2^+$ or b2, suggesting that there is no diffusible inhibitor associated with the b2 region. In addition, the superinfecting phage must carry a functional Q gene. The λb2 prophage must be cis to the bio operon for DTBS escape synthesis to occur. KAPS, the product of the bioA gene, which is transcribed in the opposite direction to that of the bioD gene,

undergoes a relatively low level of escape synthesis which is depdendent on prophage replication rather than prophage transcription. However, as with the DTBS escape, KAPS escape synthesis requires a b2 deletion in the prophage. Utilizing phages which are deleted to various extents in and near the b2 region, the block for DTBS escape synthesis has been mapped near the prophage right-hand attachment site.

Significance to Bio-medical Research and the Program of the Institute: These studies involving a system in which a virus supervenes in the control of a host function may help to elucidate virus-host interactions in other systems.

It provides information of potential value in determining the nature of oncogenic cell transformation and thus may ultimately permit the prevention of conversion of normal to tumorogenic cells. (Objective 3, Approach 2, National Cancer Program)

Proposed Course: By radioactively labeling the RNA of induced lysogens, we hope to demonstrate biochemically that prophage-induced transcription extends into the neighboring biotin operon. In addition, we will also label the DNA of these lysogens to determine whether DNA replication originating within the prophage extends into the bio operon.

Honors and Awards: None

Publications:

Krell, K., Gottesman, M.E., Parks, J. S., and Eisenberg, M. A.: Escape Synthesis of the Biotin Operon in Induced λb2 Lysogens. J. Mol. Bio., in press, 1972.

Serial No. NCI-8005
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Biochemistry of λ Int-promoted Recombination

Previous Serial Number: None

Principal Investigators: Susan Gottesman and Maxwell E. Gottesman

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total: 1 3/10
Professional: 1 3/10
Other:

Project Description:

Objectives: Phage-induced functions promote a highly efficient reaction between the DNA's of λ and E. coli. Our objective is to determine how these functions act at the biochemical level.

Methods Employed: Studies utilizing isotopically-labeled phage DNA are being carried out both in vivo and in cell-free systems. The DNA is permitted to react with extracts of cells containing phage-specific recombination functions and is then characterized by means of sucrose density gradients. The DNA products of the Int reaction are expected to have different molecular weights and sedimentation velocities than the substrate phage DNA.

Major Findings: We have succeeded in constructing bacterial strains suited for this study. These strains are deficient in DNase I and RNase I, enzymes which might interfere with our assay. In addition, the strains contain a λ prophage which hyperproduces Int-function. Incubation of high molecular weight DNA with crude extracts of these strains does not result in massive hydrolysis.

Significance: The biochemical basis of phage integration is at present completely unknown. The phage integration reaction will serve as a model system for studying the integration of oncogenic viruses in mammalian cells.

It provides information of potential value in determining the nature of oncogenic cell transformation and thus may ultimately permit the prevention of conversion of normal to tumorogenic cells. (Objective 3, Approach 2, National Cancer Program)

Proposed Course: We have now developed a system which permits us to begin to examine the biochemical basis of λ integration. We hope to use this system to detect the activity of Int and to elucidate the nature of the reaction it catalyzes.

Honors and Awards: None

Publications: None

Serial No. NCI-8006
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Control of Expression of the Galactose Operon in E. coli K12.

Previous Serial Number: None

Principal Investigator: Sankar L. Adhya

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	1
Professional:	1
Other:	

Project Description:

Objectives: The enzymes synthesized from the galactose operon are required for two purposes: Utilization of galactose and synthesis of some cellular polysaccharides. Externally added galactose induces the expression of the operon by inactivating the known gal repressor. This expression also needs cyclic AMP and its receptor protein. However, the operon must also be subject to a second control, operative particularly in the absence of external inducer, for its participation in polysaccharide synthesis. Our objective is to determine the molecular basis of both the control systems.

Methods Employed: Standard microbial genetic techniques as well as methods developed by the investigator to study specifically the galactose system using bacteriophage lambda as a tool.

Major Findings: (a) Various mutations of the gal operon - promoter defectives, insertions, ochres and operator constitutives - have been introduced into a previously isolated λpgal phage. DNA from these mutants will be used in studying the regulation of the gal operon in vitro.

(b) It has been shown previously that cyclic AMP and cyclic AMP receptor protein are required for the initiation of major transcription of the gal operon in vitro. In analogy with the lactose system, it is postulated that cyclic AMP and the receptor protein act at a site promoting the transcription (promoter) of the operon. We have characterized a mutant defective in this site. Pastan and co-workers have found that a λpgal DNA template carrying

the promoter defective mutation is largely defective in gal transcription when assayed in vitro (using purified transcription system) even in the presence of cyclic AMP and the receptor protein.

(c) Isolation and preliminary characterization of a large number of mutants, constitutive for the expression of gal operon, has revealed, in addition to previously known gal repressor defective and gal operator constitutive mutations, three new classes. All three types, although behaving differently in other respects, causes constitutive expression of the gal operon even when the operon carries the previously mentioned promoter defective mutation, suggesting at least a second control of the operon. Determination of the nature of biochemical defects in these mutants is in progress.

Significance to the Program of the Institute: In cancer cells, the expression of some genes are permanently turned on, i.e., expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using gal as a model system. This understanding might help to prevent conversions of cells to those capable of forming cancers. (Objective 3, Approach 3, National Cancer Program)

Proposed Course: 1. To determine the complete structure of the gal operon.

2. To identify all the regulatory molecules involved in turning on and off of the gal operon.

Honors and Awards: None

Publications: None

SUMMARY

Annual Report of the Laboratory of Physiology,
GL&C, National Cancer Institute
July 1, 1971 through June 30, 1972

Computational services. Computational assistance has continued to be provided for the staff of the NCI and of other institutes through programming and use of the IBM-1620 computer and through various facilities (hardware and software) available at DCRT. Applications include studies on biosynthetic mechanisms and on immunologic responses at the cellular level, statistical analyses of data, and mathematical modelling of enzyme kinetics.

Cancer physiology. Large crystals have been observed in some thyroid lysosomes in mice of the I strain. These crystals appear to cause leakage of lysosomal enzymes into the cytoplasm and may be a causative agent in carcinogenesis.

Quantitative estimates have been obtained of vesicle production and turnover of plasma membrane incidental to thyroglobulin secretion and secretion of thyroid hormones. The amount of thyroglobulin synthesized per cell per day was more than half the dry mass of the cell. If the synthesized thyroglobulin were transferred to the lumen of the follicle in microvesicles of 0.1 μ diameter, it would require approximately one microvesicle per μ^2 of cell membrane every 6 seconds. The membrane required for this process would amount to twice the dry mass of the cell per day.

Control of globin synthesis by hemin. The maintenance of the optimal rate of hemoglobin synthesis in both intact rabbit reticulocytes and in cell-free preparations is determined by the availability of hemin. New globin chains fail to be initiated when free hemin is not available. Translational repressors, inhibitors of globin chain initiation, are formed in the absence of hemin by some modification of pre-existing soluble protein of the reticulocyte. This modification may be brought about by incubation of the ribosome-free supernatant fraction of the cells, and two inhibitors have been identified, one which appears during the early period of incubation, and can be reversed by hemin, and a later inhibitor which cannot. Both appear to block globin synthesis by the same mechanism, and their action may be overcome by a ribosomal protein fraction which promotes chain initiation and ribosome dissociation into subunits. The proinhibitor as well as the irreversible inhibitor can be purified by conventional techniques, and the former can be rapidly converted to material resembling the latter with sulfhydryl reagents.

Urinary excretion patterns in leukemia. A matrix procedure for finding n best subsets from an overdetermined spectral data set used for n-component mixture analysis has been formulated and programmed. Tests run with subsets selected by this procedure and by a variety of others confirm the critical dependency of analytical results upon data selection. In worst case tests with "dirty" data the matrix

procedure finds values as good as or better than those obtained from the total set and greatly superior to those obtained by alternate selection criteria. However, the best values are confined to a single subset instead of exhibiting the expected distribution among n subsets. Additional testing is proceeding for evaluation of the procedure.

The metabolism of pseudouridine. Pseudouridine is utilized in certain strains of E. coli by the following enzymatic pathway:

(1) pseudouridine + ATP → pseudouridine-5'-P

(2) pseudouridine-5'-P ⟷ uracil + ribose-5-P

Reaction (1) is catalyzed by pseudouridine kinase and reaction (2) is catalyzed by pseudouridylate synthetase. Both enzymes are catabolite repressible; i.e., their synthesis is markedly decreased when glucose is present in the growth medium and this inhibition of synthesis can be overcome by the addition of cyclic AMP to the medium. The synthesis of pseudouridine kinase but not that of pseudouridylate synthetase is increased when pseudouridine is present in the growth medium. This result indicates that pseudouridine is an inducer of the kinase and that the two enzymes (pseudouridine kinase and pseudouridylate synthetase) are not in the same operon.

Collagen synthesis. We are studying the regulation of the extracellular protein, collagen. This protein is of interest since it is the product of a differentiated cell and there are several modifications which occur after protein synthesis which may serve as points for regulation of its secretion. We have found that the relative rate of synthesis of collagen is increased in bone tissue but not in skin during chick embryogenesis. The maximal increase occurs just prior to onset of calcification. Since calcium phosphate deposition occurs on a collagen matrix, an increased rate of collagen synthesis may be prerequisite for bone formation. We have also found that cellular structures called microtubules are involved in collagen secretion. Microtubules are involved in the intracellular transport of materials such as hormones which occur in granules or vesicles. Our experiments suggest that after collagen is synthesized on polysomes it is transported through the cells in vesicles. We have also found that although collagen is a differentiated cell product, it is synthesized throughout the growth stages of a cell parallel to the synthesis of other cellular proteins.

Energy metabolism. The work of the laboratory has been on whole animal regulatory physiology with particular reference to regulation of energy exchange and water exchange and control of food and water intake. The interaction and interdependence between metabolic and behavioral responses is of particular interest. The changes in all of these have been studied in normal rats and during the cachexia that develops with growth of a tumor.

The partition of energy expenditure in relation to total motor activity, completed for the Walker tumor (Cancer Res. 31: 98-107, 1971), has been extended to two other transplantable tumors (paper in preparation). The effect of sodium depletion on the development of cachexia has been thoroughly re-examined in the light of this new approach and no evidence has been found to support the view that sodium depletion is an important contributor (paper in preparation). The effect on the development of cachexia of imposed motor work coupled, variously, to feeding or drinking is being examined (Physiol. Behav. 8: 5-10, 1972). Early and massive failure of tumor-bearing rats to respond normally to bulk dilution of food has been demonstrated (Cancer Res. 32: May, 1972). Strain differences among rats in this and other metabolic and behavioral responses have been identified and are being pursued (paper in preparation). A quantitative method for assessment of feeding behavior independently from food intake has been developed (paper in preparation) and preliminary results on the immediacy and sensitivity of this measure in detecting systemic response to tumors have been obtained.

Radiation biology. Recovery from sublethal irradiation (LD 1%, 28 days) in terms of ability to survive a second whole-body exposure amounted to approximately 80% of the dose in 15 days. With the marrow shielded during the second exposure, recovery had occurred in 6 days, while in 15 days tolerance exceeded that of mice given no previous irradiation. This is probably attributable to the overshoot in proliferative activity in the intestinal epithelium which follows the initial irradiation. Early depression in activity is associated with ability to tolerate vinblastine, but this disappears in a few hours. Sensitivity becomes markedly increased some time before new mitotic figures are seen in the crypts. Shielding the abdomen protects against this sensitivity. When given to mice bearing lymphosarcoma P-1798 during the short post-irradiation period of low sensitivity, vinblastine has reduced lethality for the mice but retains its toxic effect on the tumor.

Immunogenetic factors affecting bone marrow transplantation in irradiated mice: Modifying the functional capacity of bone marrow cells. A degree of control over the antigen recognition system was achieved as a result of the investigation of the maternal influence on the phenotypic expression of tissue antigens and on the phenotypic expression of responsiveness. This naturally-occurring basic biological mechanism whereby the initial step in the immune response may be controlled establishes an equilibrium between antigenicity and responsiveness and apparently evolved as an immunologic mechanism to prevent rejection of the hybrid fetus. Experimentally, a graded response from unresponsiveness to sensitization may be achieved by preincubating bone marrow cells with tissue antigens prior to inoculation into lethally irradiated recipients. Among the factors controlling the degree of responsiveness are the time and temperature during incubation and the genetic disparity between the marrow cells and the tissue antigens. Both quantitative and qualitative degrees of antigenicity were demonstrable in this test system which indicates that this is a sensitive method for studying antigen recognition and the immune response.

Molecular radiation biology. Studies of the effects of ionizing radiation on nucleic acids, proteins and their constituents have been continued. Free radicals play an important role in the chain of events which leads to loss of biological activity when these macromolecules are irradiated. We have observed that the addition of small amounts of certain paramagnetic metal ions of the transition and rare earth elements in the ratio of 1 metal ion per 10 nucleotides can bring about a 5-10 fold reduction in the production of free radicals when DNA is irradiated with gamma-rays. Diamagnetic ions have no significant effect. The observed effects with certain ions are so large that they can be clearly demonstrated even when the metal to nucleotide ratio is only 1:200. The present results show interesting similarities to the quenching of the ultraviolet-induced phosphorescence in DNA and polyadenylic acid which has been explained in terms of a triplet exciton model in which the diffusion length is at least 100 nucleotide residues. The metal ions may also reduce the radical yield in γ-irradiated DNA by scavenging electrons and positive ions. Whichever mechanism applies, these experimental results indicate that either energy or charge can migrate over long distances in ordered biological structures and that such processes are of significance in localizing the absorbed radiation energy.

Similar effects of paramagnetic ions on the hydrogen transfer from exchangeable sites (those hydrogens bound to oxygen and nitrogen) to carbon-bound sites in γ-irradiated DNA were also observed. This hydrogen transfer has been shown to occur predominantly during the transformation of the neutral radicals stable at $196^{\circ}K$ to the secondary radicals stable at room temperature.

Serial No. NCI-905
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Iodide-Concentrating Mechanism of the Thyroid Gland

Previous Serial Number: Same

Principal Investigator: Seymour H. Wollman

Other Investigator: Dr. Lois W. Tice, Laboratory of Experimental Pathology, National Institute of Arthritis and Metabolic Diseases, NIH, Bethesda, Md. (Serial No. NIAMD, LEP-10)

Man Years:

Total:	1.5
Professional:	1.0
Others:	0.5

Project Description:

Objectives: To learn about (a) the mechanism by which the thyroid gland maintains a concentration of iodide elevated above that in the blood, and (b) the mechanism by which the thyroid gland accumulates protein-bound iodide.

Major Findings: Analysis has been made of autoradiographs showing the localization of radioiodide in individual follicles at various stages of the equilibration of radioiodide in a thyroid gland in which iodination is blocked. It was found that there is great heterogeneity in the rates of equilibration and that the rate depends primarily on follicle size. The large range of follicle sizes might be expected to cause great complexity in the equilibration for the whole gland. In contrast, simple results are observed equivalent to what might be expected if the gland were homogeneous. Simplification appears to be due to the fact that although the range of follicle sizes is large total volume and radioiodide content of the smaller follicles is negligible so that the range of follicle sizes which make a significant quantitative contribution to the gland is fairly limited.

Significance to Biomedical Research and the Program of the Institute: Studies of this kind provide a more detailed understanding of the mechanism of formation of thyroid hormone than heretofore possible. In addition, the studies provide insights into factors controlling the uptake of radioiodine, a radioisotope used in radiation therapy. The study could be classified under Objective 6, Approach 1: Develop methodology of radiation therapy useful in palliation of cancer.

Proposed Course of Project: To be continued.

Publications:

Nève, P. and Wollman, S. H.: Ultrastructure of the thyroid gland of the cream hamster. Anat. Rec. 171: 81-96, 1971.

Tice, L. W. and Wollman, S. H.: Ultrastructural localization of peroxidase activity on some membranes of the typical thyroid epithelial cell. Lab. Invest. 26: 63-73, 1972.

Wollman, S. H.: Histologic techniques in investigation of the thyroid. In Berson, S. et al. (Eds.): Methods in Investigative and Diagnostic Endocringolgy. Amsterdam, North Holland Publishing Co., in press.

Serial No. NCI-906
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Production and Properties of Transplantable Thyroid Tumors

Previous Serial Number: Same

Principal Investigator: Seymour H. Wollman

Other Investigator: Dr. Pierre Nève, Laboratory of Experimental Medicine, Free University of Brussels, Belgium

Man Years:

Total:	0.5
Professional:	0.5
Others:	0

Project Description:

Objectives: To study thyroid growth and the production and properties of transplantable thyroid tumors in the rat: changes occurring in the thyroid during growth, hyperplasia and involution; histologic types of thyroid tumors and their progression, and the correlation of histology with the biochemistry and physiology of the tumors.

Major Findings: The thyroid gland of mice of the I strain contains large crystals in some lysosomes. The typical lysosomal matrix is missing from many of these lysosomes. The lysosomal membranes around such crystals are frequently interrupted whereas other membranes are intact. It appears likely that the lysosomal enzymes have escaped into the cytoplasm of the cell. Allison and Mallucci have reported that intracellular release of lysosomal enzymes is associated with chromosome damage, such as chromosome breaks, and have speculated that this might be a mechanism for carcinogenesis. Crystals in lysosomes may therefore provide a mechanism by which lysosomal enzymes are released and possibly may play a role in carcinogenesis in some animals.

Significance to Biomedical Research and the Program of the Institute: The present studies on the occurrence of crystals in lysosomes may be classified as part of Objective 1, to recognize possible carcinogenic agents.

Proposed Course of Project: To be continued.

Publications:

Wollman, S. H. and Nève, P.: Postnatal development and properties of ultimobranchial follicles in the rat thyroid. Anat. Rec. 171: 247-258, 1971.

Nève, P. and Wollman, S. H.: Fine structure of ultimobranchial follicles in the thyroid gland of the rat. Anat. Rec. 171: 259-272, 1971.

Nève, P. and Wollman, S. H.: Fine structure of a fifth type of epithelial cell in the thyroid gland of the C3H mouse. Anat. Rec. 172: 37-43, 1972.

Serial No. NCI-928
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Intracellular Colloid Droplets

Previous Serial Number: Same

Principal Investigator: Seymour H. Wollman

Other Investigator: Dr. Joseph E. Loewenstein, Department of Medicine, LSU School of Medicine, Shreveport, Louisiana

Man Years:

Total:	0.5
Professional:	0.5
Others:	0

Project Description:

Objectives: To study the properties of intracellular colloid droplets in the thyroid gland.

Major Findings: Newly-formed thyroglobulin is present in apical vesicles and is secreted into the lumen of the thyroid follicle when the apical vesicle fuses with the apical plasma membrane of the follicular cell. Conversely, the process of thyroxine secretion involves phagocytosis of the luminal thyroglobulin, its intracellular digestion, followed by release of the free thyroxine into the blood.

Estimates have now been made of the rate of formation of thyroglobulin, apical vesicles and colloid droplets and the rate of turnover of plasma membrane necessary to account for the turnover of radioiodine-labeled thyroglobulin. This has been done by the analysis of quantitative autoradiography of follicles of known size, at various stages of equilibrium labeling with radioiodine. The amount of thyroglobulin made per cell per day equals half the dry weight of the cell. If this is carried to the lumen in apical vesicles of 0.1μ diameter, approximately one vesicle must discharge its contents into the lumen per μ^2 of apical plasma membrane every six seconds. The amount of membrane deposited in plasma membrane during this process must be approximately 12 times the area of apical plasma membrane per hour or twice the dry mass of the cell per day. Each cell forms about 20 droplets per hour and this takes away (by a process of bulk transfer)

about 10% of the membrane deposited by the apical vesicles. The remainder is probably removed by migration of subunits from the plasma membrane into the cytoplasm. There is no evidence as to whether membrane is formed in the Golgi apparatus from newly synthesized subunits or whether there is extensive recirculation and reutilization of subunits.

Significance to Biomedical Research and the Program of the Institute: This study indicating high rates of turnover of plasma membrane may be classified under Objective 3, Approach 5, since it has bearing on properties of cell membranes. It may also be classified under Objective 6, Approach 1, since it has to do with factors controlling the retention of a radioisotope in the thyroid and therefore has bearing on radioisotope therapy.

Proposed Course of Project: To be continued.

Serial No. NCI-919
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Urinary Excretion Patterns in Leukemia

Previous Serial Number: Same

Principal Investigator: James C. Reid

Man Years:

Total:	1
Professional:	1
Others:	0

Project Description:

Objectives: This program is a developmental effort to expand the scope of urinary analysis. Its objectives are the development of a methodology to broaden greatly the chemical information which can be extracted from the urine and the evaluation of such information for potential utility from both the clinical and research standpoints. Experimentally, a search is being conducted for significant abnormalities in the excretion patterns of all detectable ultraviolet-absorbing components of a set of urine specimens collected from leukemic and control subjects. Such components include various nucleic acid congeners but are not limited thereto.

Methodology: The basic concept is that it may be feasible to construct an analytical system powerful enough to deal with the complexity of urinary composition by the application of a computational technique for multi-component mixture analysis to urinary fractions obtained after preliminary chromatographic simplification. The data required are simply absorbance spectra, which can be obtained by a single non-specific instrumental procedure. The necessity for the application of many chemical procedures, each specific for a particular component, is eliminated thereby and the attendant reduction of labor would make it feasible to study many components simultaneously, i.e. to study patterns of excretion. Moreover, techniques which have been developed in this program permit quantitative estimation of the components without the necessity for chemical identification, a feature which facilitates further the recognition of pattern abnormalities if they should occur.

The preliminary chromatographic simplification is effected by a system of ion-exchange column procedures developed earlier in the course of this

program. The system reduces each urine specimen to approximately fifty fractions. Each fraction is a mixture whose components typically number about a half-dozen, and in general the individual components can be obtained in small amounts by paper chromatography. The absorbance spectra of the isolated components then serve as the basis for computing the composition of the corresponding fraction obtained from other urinary specimens.

Status:

1. During the current period the work has continued to focus on standardization of computational technique and particularly on the problem of selecting the best subset from an overdetermined spectral data set used for multicomponent mixture analysis. A selection technique based upon maximization of matrix determinants has been programmed and incorporated into the main software system. Tentatively, it is expected that this will be the technique finally selected as the basis for a standardized procedure. It generates n matrices and n solution vectors for an n-component system and it is based upon a heuristic expectation that each matrix will give a best value for one component. Moreover, variation between the solution vectors affords an additional index of overall data quality since in the ideal case all would be identical.

2. Utilizing utility software previously written for the 1620 plotter, a graphic output section has been programmed and incorporated into the main system. Graphic output presents many features of an analytical computation in compact form and complements other system facilities for error analysis.

3. In worst case tests of "dirty" data the matrix-based selection technique yields solution vectors as good as or better than those obtained by the use of the total data set and greatly superior to those obtained by other selection criteria. The importance of proper data selection is thereby confirmed. In addition, the variation between "best" solutions is about the same as the variation between analyses performed by alternate chromatographic methods. However, best values tend to be confined to a single subset of data rather than being distributed among n subsets as expected. Additional testing is proceeding for evaluation of the procedure.

Significance to Biomedical Research and the Program of the Institute: National Cancer Plan objective #5 (diagnosis), approach #4.

Proposed Course: Further testing of the new software will continue for the purpose of finalizing the form of the computational technique and means for distribution of residual error will be investigated. In addition, software will be written for production of plotter-drawn diagrams integrating the clinical chromatographic profiles with their associated spectral entities. An earlier attempt to produce such diagrams with the plotter then in use yielded diagrams whose quality is inadequate to serve as journal plates.

Serial No. NCI-925
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Protein Synthesis and Its Control in Normal and Tumor Cells

Previous Serial Number: Same

Principal Investigator: Marco Rabinovitz

Other Investigators: Dr. Martin Gross, Research Associate;
Dr. Satoshi Mizuno, Visiting Fellow; and
Dr. Kenneth Balkow, Guest Worker.
Also studies in collaboration with Drs. T. R. Breitman and C. R. Maxwell.

Man Years:

Total:	6.5
Professional:	4.0
Others:	2.5

Project Description:

Objectives:

a) To study, in both normal and tumor cells the controls exercised by these cells in regulating the synthesis of protein. This is generally made through an analysis of the biochemical lesions introduced within the pathway by deficiencies of essential nutrients or by inhibitors.

b) Where applicable, to extend the information obtained in the first objective to the tumor-bearing animal under controlled nutritional conditions, with the view of specific inhibition of protein synthesis by cancer cells or to other selective lethal effects.

Methods Employed:

a) Organic synthetic techniques are developed for preparation of potentially effective amino acid antagonists.

b) Incorporation of labeled amino acids into protein of isolated cells or cell-free systems is measured. Such studies involve radioactive counting techniques and stripping film radioautography. Respiration and anaerobic glycolysis may be followed by manometric techniques.

c) Subcellular components are isolated by differential and zonal centrifugation. These components are further fractionated by extraction, hydrolysis or chromatographic techniques. The characteristics of the block in amino acid utilization within these fractions are evaluated.

Major Findings:

Our principal efforts have been directed toward outlining the mechanisms of formation and action of translational repressors which regulate the synthesis of globin with the availability of hemin. Previous studies in this laboratory have demonstrated that a repressor is formed from pre-existing material (proinhibitor) and that this transformation is blocked by hemin.

1. Formation of the Translational Repressor (in collaboration with Dr. Martin Gross)

Two forms of the repressor have now been detected, an early form which can be inactivated by hemin (reversible inhibitor) and a later form which cannot. The formation of both the reversible and irreversible inhibitors is blocked by hemin.

Both the proinhibitor and irreversible inhibitor are protein complexes of about 3×10^5 mol. wt. The proinhibitor can be freed of smaller molecules, including hemoglobin, and converted to both reversible and irreversible inhibitors by incubation in the absence of hemin. This conversion of proinhibitor to inhibitory material resembling the natural repressors is markedly accelerated by sulphydryl reagents, such as N-ethylmaleimide and o-iodosobenzoate. The results suggest that the conversion of proinhibitor to inhibitor may involve a conformational change.

The irreversible inhibitor is being purified by standard protein isolation techniques as a prelude to its labeling and determination of its binding characteristics and mechanism of action.

2. Action of a Ribosomal Factor in Overcoming the Action of the Repressor (in collaboration with Dr. Satoshi Mizuno)

An initiation factor which is extracted from reticulocyte ribosomes by 0.55 M KCl and is eluted from a DEAE column with 0.16 M KCl can overcome the action of the repressor. These results suggest that the repressor may act by antagonizing the activity of the factor on the ribosome. This, however, is not the case. When the factor is assayed directly in either of two systems,

(1) a KCl extracted ribosome system requiring the factor for protein synthesis,

(2) as a dissociation factor which promotes the formation of ribosomal subunits from 80S ribosomes,

its activity is not inhibited by very large levels of the repressor. We are currently attempting to explain the cause of this apparent anomaly.

3. Status of tRNA Bound to Ribosomes in Control and Repressed Systems (in collaboration with Dr. Kenneth Balkow)

During protein synthesis, aminoacyl-tRNA molecules are bound to the ribosome-polyribosome complex in order of amino acid insertion into the polypeptide chain. The material bound to purified ribosomes can be assayed after extraction by charging with the appropriate amino acid. The binding of methionyl-tRNA to monoribosomes and ribosomal subunits is not decreased in repressed systems. These results suggest that the block in globin chain initiation may be at a locus after the initial methionyl-tRNA association.

As an adjunct to this study, it was found when protein synthesis is blocked by appropriate antagonists which prevent charging of tRNA molecules with an amino acid, the uncharged tRNA binds to the messenger RNA-ribosome complex. These results are contrary to expectations suggested from derived partial systems in which only charged tRNA is presumed to bind.

Significance to Biomedical Research and the Program of the Institute: These studies are designed to help explain the nature of the lesions caused by nutritional deficiencies and antagonisms at the level of macromolecular biosynthesis. Such evaluations should provide a basis for our understanding of control of protein-forming systems and cell types. This project conforms to the NCI research plan as follows: Objective 3, to prevent conversions of cells to those capable of forming cancers; Approach 1, nature and modification of precancerous state and determination of mechanisms accounting for high degrees of stability of cell functioning.

Proposed Course of Project: Those areas which appear most promising will be investigated.

Publications:

Rabinovitz, M.: Some recent advances in hemoglobin biosynthesis. Ann. of Clin. Lab. Sci. 1:10-13, 1971.

Freedman, M. L., and Rabinovitz, M.: Structure of the large β-chain polyribosome of rabbit reticulocytes rendered isoleucine-deficient by o-methylthreonine. Molec. Pharmacol. 7: 317-322, 1971.

Maxwell, C. R., Kamper, C. S., and Rabinovitz, M.: Hemin control of globin synthesis: An assay for the inhibitor formed in the absence of hemin and some characteristics of its formation. J. Molec. Biol. 58: 317-327, 1971.

Serial No. NCI-946
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Role of Hemin in the Control of Globin Synthesis

Previous Serial Number: Same

Principal Investigator: Charles R. Maxwell

Other Investigator: Marco Rabinovitz

Man Years:

Total:	2
Professional:	1
Others:	1

Project Description:

Objectives:

This investigation is to determine the mechanism by which hemin stimulates in vitro synthesis of globin in a cell-free system prepared from rabbit reticulocytes.

Major Findings: None. Minor findings have been turned over to Dr. Rabinovitz for incorporation in future reports.

Significance to Biomedical Research and the Program of the Institute: The knowledge of the basic control mechanism in any cell is of potential basic importance to cancer research when one considers that one simple-minded way of looking at a cancer cell is that it is a cell with a disfunction in its normal control mechanism.

National Cancer Institute research plan: Objective 3, Cause and prevention. Approach 1, Nature and modification of precancerous state and determination of mechanisms accounting for high degrees of stability of cell functioning.

Proposed Course of Project: I am retiring; project will be terminated.

Publications:

Maxwell, C. R., and Mencken, C.: Use of high-performance swinging-bucket rotors for micro preparative centrifugation. Anal. Biochem. 44: 235-238, 1971.

Serial No. NCI-944(a)
1. Laboratory of Physiology
2. Energy Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Total Metabolism

Previous Serial Number: Same

Principal Investigator: Seoras D. Morrison

Man Years:

Total:	5
Professional:	2
Others:	3

Project Description:

Objectives:

(a) Investigation of the patterns of heat production of rats with concomitant recording of feeding behavior and other activity with a view to finding how the energy expenditure, feeding and general behavior pattern are related to tumor growth and to finding the nature of the loads imposed by a tumor on the host.

(b) Investigation of relative changes in water and material exchanges in tissue compartments of the rat during imposed and induced changes in food and water intake and the relation of these to tumor induction and growth.

(c) Examination of possible experimental methods of simulating in non-tumor-bearing rats the cachexia and decline of food intake found during neoplastic growth.

(d) Development of quantitative and reproducible assessment of behavioral output, to yield more sensitive and earlier detection of systemic effects of tumors.

Methods Employed: The methods of indirect calorimetry, operant conditional responses, placement of electrolytic lesions in or stimulation of the central nervous system, continuous or programmed infusions into unrestrained animals, and methods of orthodox nutritional studies. Ballistic methods of recording and partitioning total motor activity. Computer methods of numerical analysis of serial records of tumor growth and of continuous

records of gaseous exchange. Sprague-Dawley rats and Buffalo rats are used in all animal experiments. Most tumor-bearing rats are inoculated with Walker 256 carcinoma, but other transplantable tumors are also used.

Major Findings:

(a) Compartmentation of energy expenditure, established and analysed for the Walker tumor (Cancer Res. 31: 98-107, 1971) has been extended to two other transplantable tumors (paper in preparation).

This principle has been applied to the apparent restoration of food intake after Na loading of the host. It was found that Na only rarely restores food intake and it restores bodyweight by water retention (paper in preparation).

A systematic examination has been started of the quantitative changes, induced by a tumor, in the response to stimuli known to modify food intake of normal animals. The Walker tumor severely depresses the response to bulk dilution of food (Cancer Res. 32: May 1972, in press). There is no qualitative change in the response to hypoglycemia but there may be a quantitative change.

(b) Work is continuing on the extent to which water intake and food intake during tumor growth can be modified by coupling a work requirement to drinking. The increase in water intake is eliminated by minimal coupled work (Physiol. Behav. 8: 5-10, 1972). The effect on food intake is being studied (preliminary paper in preparation).

(c) Work on depression of food intake by infusion of lactic acid is continuing.

Major differences have been found among strains of rats in metabolic and behavioral responses to stimuli (paper in preparation). The extent to which these idiosyncracies of host strain can account for observed differences in response to tumors is being investigated.

(d) The quantitative measurement and partitioning of different forms of behavioral activity has been found to be feasible.

Studies on the measurement of feeding activity independently of the measure of food intake are under way and are allowing much more detailed analysis of aspects of control of food intake (paper in preparation). Preliminary extension of this work to tumor growth shows detectable effects on feeding efficiency in the earliest stages of tumor growth.

Sensitivity to bulk dilution of food appears very early in tumor growth.

Significance to Biomedical Research and the Program of the Institute: The findings on partition of energy expenditure and of tissue compartments and on processes influencing food and water intake make it possible to

examine some features of tumor-host relationships that have not previously been subject to analysis. The methods and findings on sensitive and early changes in systemic response to tumors should be utilizable in refined animal testing of therapeutic agents. It is believed that the work is throwing useful light on the reasons for the rapid and extreme debilitation of the host during tumor growth. The section of the National Cancer Plan that the work most closely approximates is: Rehabilitation, Objective 7 (To restore patients with residual deficit as a consequence of their disease or treatment to as nearly a normal functioning state as possible). Approach 1 (Develop improved means to provide treatment which will encompass physiological aspects of cancer).

Proposed Course of Project: Work on energy and water exchange of tumor bearers will be continued along with study on the interaction of metabolic and behavioral responses of normal and cancerous animals, with particular reference to the reasons for the changes in food and water intake and depletion of host tissues that occur during tumor growth.

Publications:

Morrison, S. D.: Work and water intake and the water need of rats during growth of a tumor. Physiol. Behav. 8: 5-10, 1972.

Morrison, S. D.: Feeding response to change in absorbable food fraction during growth of Walker 256 carcinosarcoma. Cancer Res. 32: May, 1972.

Serial No. NCI-944(b)
1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mathematical Programming

Previous Serial Number: Same

Principal Investigator: Florence K. Millar

Man Years:

Total:	1
Professional:	1
Others:	0

Project Description:

Objectives: To facilitate the investigation of bio-medical research problems through the application of computer programming and computer data processing. Many of the projects reported here are collaborative efforts with investigators in other units of NCI and NIH.

Methods Employed: The IBM-1620 facility of the NCI has been described in previous reports [see NCI-944(b), 1969]. For some computer applications, use has been made of the central NIH computer facility (Computation and Data Processing Branch, DCRT).

Major Findings:

Computer Operations:

The 1620 has been operated as in the past on a one-shift basis with prime time shared by Drs. Reid and Millar and with use by other investigators (see below). Computer operations include routine procurement of supplies, scheduling of user time and maintenance, and making additions to the system software as needed.

The on-line CalComp plotter attachment has continued to provide members of the staff with an automatic, flexible method of visualizing their calculated data. Additional programs have been developed, e.g., for plotting normalized results or isotopic ratios as in double-label experiments.

Computer Applications:

(1) Processing of data from liquid scintillation spectrometers.

The 1620 is used to reduce and process the raw data from single- and double-label experiments in a variety of bio-chemical studies as follows:

Activation of the immune lymphocyte by antigens and mitogens (Dr. S. A. Rosenberg, Dr. R. Levy, C/I)

An assay for anti-tumor cytotaxic lymphoid cell activity based on the in vivo elimination of ^{125}I-labelled tumor cells (Dr. W. J. Martin, C/I)

Comparison of isoaccepting species of tRNA isolated from normal and malignant cells (Dr. J. F. Mushinski, C/LCBGY)

Effect of steroids on tRNA synthesis in HTC cells (Dr. E. I. B. Thompson, Dr. M. Lippman, C/LB)

Analysis of arbovirus proteins and RNA species (Dr. J. G. Levin, C/LP)

Distribution of rare nucleosides in tRNA (Dr. A. Peterkofsky, H/LBG)

(2) Statistical analysis and curve fitting.

Standard statistical methods, including simple linear and multiple regression analysis, have been applied. In studies of enzyme kinetics, use has been made of programs developed by Cleland (Nature 198: 463, 1963) and of other curve-fitting techniques available at the DCRT to handle the non-linearity of the reaction equations.

Studies for which the computer has application in the general area of statistics and curve fitting are as follows:

Relationships among components of material exchange measured during tumor growth (Dr. S. D. Morrison, C/LPHY)

Effect of ionizing radiation on DNA, protein, and their constituents (Dr. P. Riesz, Dr. E. Rotlevi, Mr. H. M. Moss, C/LPHY)

Statistical parameters in radiation studies (Dr. W. W. Smith, Ms. D. E. Uphoff, C/LPHY)

Simulation of ESR spectra from parameters (Program obtained from external source) (Dr. E. Rotlevi, C/LPHY)

Kinetic studies on UMP-CMP kinase (Dr. E. P. Anderson, Dr. T. Q. Garvey, C/LB)

Measurement of cyclic AMP levels in tissues (Dr. A. Peterkofsky, Ms. C. Gazdar, H/LBG, Dr. J. S. Stoff, H/LKEM, Dr. V. Manganiello, H/MD)

Multiple regression program testing (Mr. D. E. Towne, CH/SINFC)

(3) Processing of data from energy metabolism studies.

Preliminary programming has been done for the analysis of paper tape output from the data acquisition system to be used with the small animal calorimeter (Dr. S. D. Morrison, C/LPHY)

Significance to Biomedical Research and the Program of the Institute: Mathematical and computer programming applications contribute to the investigation of research problems by providing (1) accurate and efficient methods for handling laborious calculations of data and visualizing results, (2) the ability to store and retrieve large amounts of data, and (3) opportunities for testing mathematical models based on experimental results. This project most closely supports Objective 4, Approach 5 of the "National Cancer Plan" by providing methods of developing mathematical models of the cancerous process.

Proposed Course of Study: Computational techniques and programs will continue to be developed and applied in response to computer-related needs of the scientific staff.

Serial No. NCI-945
1. Laboratory of Physiology
2. Physical Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of the Factors Regulating the Synthesis of Collagen during Embryonic Development and Growth of Cultured Fibroblasts

Previous Serial Number: Same

Principal Investigator: Beverly Peterkofsky

Other Investigator: Robert Diegelmann, PHS Fellow, Guest Worker

Man Years:

Total:	3.5
Professional:	2
Others:	1.5

Project Description:

Objectives: To investigate the factors controlling the synthesis and secretion of the structural protein collagen, during embryonic development and during the growth of cultured fibroblasts.

Major Findings:

I. Tyrosine aminotransferase

Acid phosphatases from chick liver were partially purified on DEAE cellulose. Several peaks of phosphatase were detected but the specificity towards tyrosine aminotransferase (inactivation) differed. One peak especially had a very high preference for inactivation of tyrosine aminotransferase relative to dephosphorylation of dinitrophenyl phosphate.

II. Collagen

A. Isolated connective tissues labeled with precursors in ovo.

We had previously shown that when embryonated eggs were injected with [^{14}C]-proline, and the rate of incorporation into collagen relative to other proteins was measured using collagenase degradation as an assay, that the rate increased in bone tissue but not in skin. We have now measured

Calcium-45 deposition into bone by injecting [Ca^{45}]Cl_2 into embryonated eggs at various stages of development, removing isolated tissues, and measuring the isotope in insoluble $CaPO_4$. The rate of calcification was determined from a 30 min. interval during the linear uptake of the labeled compound. The rate of calcification increased markedly in both frontal bone and lower limb (tibiae and fibulae) after the rate of collagen synthesis had increased to a maximum, suggesting that in bone tissue, collagen synthesis increases in order to provide sufficient matrix for calcium phosphate deposition and in this way may help to regulate differentiation of bone cells.

B. In vitro Studies with frontal bone.

Frontal bones were removed from embryos varying from 11 to 17 days of development. These were incubated in tissue culture medium containing [^{14}C]-proline for 90 mins. and the relative rate of collagen synthesis was determined. An increasing rate of collagen synthesis with increasing development was observed which exactly paralleled that observed after in ovo labeling. These results indicate that the factor or factors responsible for the change are present in the bone itself rather than being a transient effect of other organs in the embryo.

C. Collagen Synthesis in Cultured Fibroblasts.

Further studies have been carried out to determine whether the differentiated function of collagen synthesis occurs throughout the various stages of growth of several lines of fibroblasts. It has been postulated that differentiated functions are expressed only in non-growing cells. The assay which we use to measure collagen (degradation by collagenase) has the advantage of being able to detect collagen molecules in which proline residues may be hydroxylated or not, while previous assays relied on the measurement of hydroxyproline and thus could only measure the hydroxylated form of collagen. Our results show that in L-929 cells, collagen is synthesized throughout the growth cycle at the same relative rate, but in the absence of ascorbic acid, which is a cofactor for proline hydroxylase, the collagen is unhydroxylated and therefore would not be detected by other procedures. We also found that collagen synthesis occurs at approximately the same relative rate in growing and non-growing cells of the lines Balb 3T3, 3T6 and 3T12 and Swiss 3T6. A line of Kirsten Sarcoma transformed 3T3 synthesized collagen at one-fifth the rate as the parent 3T3.

D. Collagen Secretion.

Collagen is normally found extracellularly in animals and thus one of the steps at which its synthesis may be controlled is secretion from the cell. We have studied this process using frontal bones incubated in vitro and 3T3 cells in culture. When frontal bones were incubated for 90 min. with [^{14}C]-proline, the isotope was incorporated into collagen, of

which 15-25% was found in the medium. In 3T3 cells, 60% of the collagen labeled by [^{14}C]-proline was found in the medium after 2 hours, the remainder being retained in the cells. Secretion in both systems was inhibited by treatment of the bones or cells with the microtubular disruptive drugs colchicine and vinblastine, suggesting that microtubules are involved in collagen secretion. Since most systems in which secretion is inhibited by these agents involve the storage or movement of materials in secretory vesicles or storage granules, our results suggest that collagen is secreted in a similar manner. The only previous evidence for the involvement of vesicles in collagen secretion has been from electron micrography, the interpretation of which was ambiguous.

Significance to Biomedical Research and the Program of the Institute: Approach 19-1-1. Nature and modification of the precancerous state and determination of mechanisms accounting for high degrees of stability of cell functioning. Objective 3. To prevent conversion of cells to those capable of forming cancers.

Proposed Course of Research:

I. Tyrosine aminotransferase

This project has been discontinued.

II. Collagen

A. Identification of factors responsible for the developmental increase in collagen synthesis.

Since the developmental increase in collagen synthesis can be observed in bones labeled in vitro, the factor or factors responsible reside in the bone itself when it is removed from the embryo. We are attempting to determine what this factor or facotrs may be by three different approaches: 1) determine whether bone tissue contains either low molecular weight compounds or macromolecules which will influence collagen synthesis in bones incubated in vitro 2) prepare cell-free protein synthesizing systems derived from bones of various aged embryos in order to determine whether polysomes or soluble factors may be rate limiting for collagen synthesis 3) set up organ cultures of frontal bones from 11 day old embryos. In such a system, developmental changes would occur in culture and it would be possible to study the effect of inhibitors such as actinomycin D, which would reveal whether increases in messenger RNA were responsible for increased collagen synthesis.

B. Collagen synthesis and secretion in cultured fibroblasts.

Since 3T3 cells secrete 60-70% of the collagen which they synthesize, this is an excellent system to study the secretion process. Previous

investigators have shown that when hydroxylation of proline and lysine in collagen is inhibited by the iron chelator αα-dipyridyl, collagen secretion is almost completely inhibited. The use of this inhibitor, however, may be affecting secretion at some other site. We are studying the effect of hydroxylation on secretion by using ascorbic acid deprivation as a means of producing unhydroxylated collagen. Since our assay procedure measures both unhydroxylated and hydroxylated collagen, there is no problem in measuring collagen synthesis in the absence of ascorbic acid. Preliminary results indicate that the rate of collagen secretion is reduced but not completely suppressed in the absence of the vitamin. In addition, we have initiated studies on the effect of dibutyryl cyclic AMP on collagen synthesis in sarcoma transformed 3T3 cells, since it has been reported that this compound has a reverting type of effect on transformed cells.

Publications:

Peterkofsky, B. The inactivation of chick tyrosine aminotransferase by acid and alkaline phosphatases. Biochem. Biophys. Res. Commun. 43: 1171-1178, 1971.

Diegelmann, R. F. and Peterkofsky, B. Inhibition of collagen secretion from bone and cultured fibroblasts by microtubular disruptive drugs. Proc. Nat. Acad. Sci., U.S.A., 69: 892-896, 1972.

Diegelmann, R. F. and Peterkofsky, B. Collagen biosynthesis during connective tissue development in chick embryo. Develop. Biol. June 1972.

Serial No. NCI-948
1. Laboratory of Physiology
2. Physical Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Control of Macromolecular Synthesis

Previous Serial Number: Same

Principal Investigator: Theodore R. Breitman

Other Investigators: David R. Krauss, Research Associate; and collaboration with Marco Rabinovitz

Man Years:

Total:	4.5
Professional:	4.0
Others:	0.5

Project Description:

Objectives: To clarify metabolic transformations of nucleic acids and their nucleotide precursors.

Major Findings:

A. The Metabolism of Pseudouridine

As reported in the 1971 Annual Report, pseudouridine is metabolized in certain E. coli strains by the following coupled enzymatic reaction:

1) pseudouridine + ATP $\xrightarrow{\text{pseudouridine kinase}}$ pseudouridine-5'-P + ADP

2) pseudouridine-5'-P $\xleftrightarrow{\text{pseudouridylate synthetase}}$ uracil + ribose-5-P

Pseudouridine kinase has a pH optimum of 7, and requires ATP, Mg^{++}, and either K^{+} or NH_4^{+}. Pseudouridine kinase is distinct from uridine kinase and deoxythymidine kinase. Both pseudouridine kinase and pseudouridylate synthetase are catabolite repressible. However, pseudouridine kinase activity is induced by the presence of pseudouridine in the growth medium while pseudouridylate synthetase activity is not. This result indicates that the two activities do not share the same operon.

Purification of pseudouridylate synthetase was initiated. Through ammonium sulfate fractionation, gel filtration and DEAE column chromatography an approximately 50-fold purification has been obtained.

B. Methionineless Death in E. coli (in collaboration with M. Rabinovitz)

In previous Annual Reports (1970 and 1971) the phenomenon and biochemical basis for methionineless death was described. It was found that the DNA degradation associated with this phenomenon was triggered by the action of the E. coli strain 15 restriction enzyme (an endonuclease) which apparently recognizes unmethylated regions of the DNA. The known involvement of S-adenosylmethionine as the methyl donor for nucleic acid methylation prompted interest in methods of inhibiting its formation. Cyclomethionine was synthesized and tested as an inhibitor of the enzyme S-adenosylmethionine synthetase. It was found that it competed with methionine in this reaction only at very high concentrations.

Significance to Biomedical Research and the Program of the Institute: Findings pertaining to an elucidation of the mechanisms involved in the modification of nucleic acids are important as they may suggest possible means of therapeutic control of cellular growth. National Cancer Plan Objective #3, Approach #1.

Proposed Course:

1. Purification and characterization of pseudouridine kinase and pseudouridylate synthetase.

2. Further studies on S-adenosylmethionine synthesis and/or biological effects of S-adenosylmethionine analogues.

Publications

Solomon, L. R. and Breitman, T. R.: Pseudouridylate synthetase of Escherichia coli: A catabolite-repressible enzyme. J. Bact. 107: 535-542, 1971

Solomon, L. R. and Breitman, T. R.: Pseudouridine kinase of Escherichia coli: A new enzyme. Biochem. Biophys. Res. Commun. 44: 299-304, 1971

Breitman, T. R., Finkleman, A., and Rabinovitz, M.: Methionineless death in Escherichia coli 15. J. Bact. 108: 1168-1173, 1971

Serial No. NCI-936
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Recovery from effects of radiation or other initial treatment in terms of tolerance to subsequent treatments

Previous Serial Number: Same

Principal Investigator: Willie W. Smith

Man Years:

Total:	3
Professional:	2
Others:	1

Project Description:

Objectives: To identify and quantify effects of sublethal irradiation or chemotherapeutic treatments which alter tolerance to subsequent treatments.

Major Findings:

In mice given radiation lethal to 1% (520 rads X-ray), second exposures showed evidence of recovery from 24% of the dose in 6 hours, increasing to 80% in 15 days. For the same initial dose but with legs shielded during the second exposure, in terms of lethality mice had recovered from half of this dose in 6 hours and all of it in 6 days, while in 15 days tolerance considerably exceeded that of mice given no previous radiation.

For several hours after the sublethal irradiation, when there is little mitotic activity in intestinal crypt cells, an injection of vinblastine (5 mg/kg i.p.) was relatively well tolerated. A differential effect on tumor and host, with more than additive effects on survival, was demonstrated in mice bearing lymphoma P1798 but not L1210. Mitotic cells per crypt increased to well above normal between the first and third days after irradiation and VLB given in this period reduced the number count to essentially zero, with only an occasional mouse surviving. By the fourth day these effects were less severe. VLB given one or two days before irradiation was considerably less toxic and this schedule significantly increased survival in mice bearing lymphosarcoma P1798.

Significance to Biomedical Research and the Program of the Institute: Agents useful in cancer therapy are generally damaging to host as well as tumor, and their effectiveness is usually limited by toxicity to the host. Our research program is directed to increased understanding of the events resulting from such agents as radiation and drugs and the time-dependent interactions of treatments given sequentially. In the experimental situation it is possible to develop a physiological basis for effective therapy within the limitations of tolerance and in so doing to acquire a greater comprehension of the essential functions of particular tissues.

National Cancer Institute research plan: Objective 6, Therapy. Approach 1, Develop and utilize methodologies of chemotherapy and radiation (alone and in combinations) in the cure and palliation of cancer.

Proposed Course of Project: I plan to continue studies of effects of treatments which are of interest in cancer therapy, particularly emphasizing their influence on tolerance to further treatment and possible differential effects on tumor and host.

Serial No. NCI-941
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunogenetic factors affecting bone marrow transplantation in irradiated mice: Modifying the functional capacity of bone marrow cells.

Previous Serial Number: Same

Principal Investigator: Delta E. Uphoff

Man Years:

Total:	3.5
Professional:	1.0
Others:	2.5

Project Description:

Objectives: To develop experimental methods for improving the survival of recipients of bone marrow grafts in order to increase the feasibility of using bone marrow transplantation as a critical organ replacement procedure when chemotherapeutic agents or irradiation in the lethal dose range are required to effect a cure or for the palliation of cancer.

Methods Employed: The immunogenetic nature of this investigation requires the use of inbred strains and especially derived substrains which are only available from among the laboratory mouse populations; however, the basic mechanisms are, in all probability, common to all mammals. Survival studies on lethally-irradiated mice with marrow grafts representing different degrees of genetic disparity reflect the functional capacity of the marrow transplant. Characteristic changes in the survival patterns indicate the ability of the marrow graft to respond to: a) erythropoietic stimuli, b) various antigenic stimuli including the tissue antigens of the irradiated recipient in which the marrow graft is growing, since the graft-versus-host reaction has been the greatest single obstacle encountered in the absence of genetic uniformity, and c) altered leukemogenic processes.

Major Findings: Bone marrow transplantation was developed as a sensitive test system for investigating the antigenic recognition mechanism after it was realized that the ability of the bone marrow graft to function was changed by contact with the antigens of the recipient in which the marrow was expected to grow or by either *in vitro* or *in vivo* contact with

antigenic substances prior to its transplantation.

A naturally occurring mechanism, producing effects analogous to those found after marrow stem cells are exposed to antigens, exists as maternal influences. By nurturing her progeny, the mammalian female controls the phenotypic expression of the tissue antigens of her progeny and also influences their immune responsiveness. By investigating the nature of the basic biological phenomenon that was responsible for maternal influences, it was found that the immune response was controlled by controlling antigen recognition. Special genetic crosses and especially derived substrains of mice were used to demonstrate the extent of the maternal influence on the immune potential of subsequent generations. An unresponsive state is achieved by controlling the antigen recognition mechanism in equilibrium with a controlled phenotypic expression of tissue antigens. It was thus possible to apply the concept of graded responsiveness toward graded antigenicity to control of the homograft reaction since both quantitative and qualitative differences in tissue antigenicity were readily demonstrable with these methods. These subtle differences in antigenic expression, existing as a result of a maternal modification, control whether sensitization or unresponsiveness follows the exposure of marrow stem cells to tissue antigens.

This is a basic biological mechanism, and as such should be operable in all segments of the mammalian populations in which the developing fetus has contact with maternal antigens. In addition, the mother is potentially a more compatible donor than the father, a fact substantiated in 3 out of 4 genetic combinations tested.

Significance to Biomedical Research and the Program of the Institute: These naturally occurring immunogenetic phenomena are in part responsible for the greater success achieved under genetically controlled experimental conditions than under clinical conditions. The experimental modification of responsiveness and the induced or acquired modification of antigenicity are relevant to the problem of immunotherapy of cancer and to successful organ replacement, while the investigation of maternal influences is basic to both developmental biology and genetics.

National Cancer Institute research plan: Objective 6, Therapy. Approach 1, Develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (alone and in combinations) in the cure and palliation of cancer.

Proposed Course of Project: No change in the course of this investigation is anticipated as long as new aspects of the antigen recognition system are revealed. Other aspects of this project reported in previous years are being continued. The project will be expanded to include consideration of the mechanism by which modification may be induced.

Publications:

Uphoff, D. E.: Modified antigenicity: An immunologic mechanism for species survival. Transplant. Proc. 3: 207-209, 1971.

Uphoff, D. E.: Tolerance induction and sensitization by preincubation of bone marrow with allogeneic erythrocytes. J. Nat. Cancer Inst. 47: 1223-1231, 1971.

Uphoff, D. E. and Draper, L. R.: Functional capacity of bone marrow modified by contact with antigenic substances. J. Nat. Cancer Inst. 47: 1233-1240, 1971.

Uphoff, D. E.: Maternal modification of antigen recognition in ova-transfer substrain RIIIeB. J. Nat. Cancer Inst. 48: 517-522, 1972.

Uphoff, D. E.: Maternal modification of self recognition in the ova-transfer substrains of C3H mice. J. Nat. Cancer Inst. 48: 965-971, 1972.

Uphoff, D. E.: Maternal modification of responsiveness in the ova-transfer substrain DBA/2eB. J. Nat. Cancer Inst. 48: 1317-1322, 1972.

Serial No. NCI-942
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Effects of Ionizing Radiation on Macromolecules of Biological Importance

Previous Serial Number: Same

Principal Investigator: Peter Riesz

Other Investigator: Eitan Rotlevi

Man Years:

Total:	4.5
Professional:	3.0
Others:	1.5

Project Description:

Objectives:

The effects of ionizing and ultraviolet radiation on biological macromolecules and their constituents are being investigated. For reproductive death, DNA is the target molecule in viruses and is at least a part of the target molecule in bacteria and mammalian cells. Radiation damage to DNA is produced by the "direct effect" through the formation of radical ions, electrons, excited states and neutral free radicals or by the "indirect effect" where radical species formed in the surrounding medium by radiation react with DNA. In the case of water, these species are hydrated electrons, hydrogen atoms and hydroxyl radicals. In the case of radiation damage to the chromosome, radicals formed in the nucleohistone may cause damage to the DNA.

In the chain of events that leads to loss of biological activity, free radicals play an important role. Thus, for example, when biologically active DNA of bacteriophage ϕ X 174 is irradiated in the dry state with γ-rays under vacuum, cysteamine protects to the same extent (by about a factor of 1.6) against inactivation and the production of free radicals. We have found recently that the addition of certain metal ions to DNA in the ratio of 1 metal ion per 10 nucleotides, can reduce the radical yield by a factor of about 10.

An understanding of the mechanisms by which ionizing radiation brings about the loss of biological activity of these macromolecules is likely to help in the development of new methods for altering the efficiency of cell killing with possible benefits to radiation therapy.

Methods Employed:

When dry proteins or nucleic acids are irradiated with gamma rays or ultraviolet radiation in the absence of air (at 10^{-4} mm pressure), trapped free radicals are generated. Subsequent exposure to tritiated hydrogen sulfide or hydrogen iodide leads to the formation of carbon-tritium bonds. After removal of exchangeable tritium the protein is hydrolyzed, the amino acids are separated on an automatic amino acid analyzer and the tritium content of each amino acid is determined by means of an associated flow counter. It has been shown that the tritium distribution so obtained corresponds closely to the free radical distribution at the time of exposure to the tritiated free radical interceptor.

Trapped radicals in lyophilized proteins and nucleic acids were produced by:

(a) Gamma-radiolysis using a Co^{60} source.

(b) Photolysis with 2537 Å light using a low pressure Mercury arc. A PCQ-X1 Photochemical Lamp with 4 circular lamps was used; 80-90% of the total radiation is at 2537 Å.

(c) Electrical discharge was generated by a high frequency induction coil (Tesla Type) adjusted for maximum voltage (40-50 kv).

Electron Spin Resonance studies were carried out at Walter Reed Army Medical Center using the Varian E-9 ESR Spectrometer located in the laboratory of Dr. E. S. Copeland.

Proteins and DNA were tritium labeled on exchangeable sites by exposing the dried materials to tritiated water vapor (5 curie/gm) at 20 torr for 10 minutes in a high vacuum system located in a hood in the Isotope Laboratory (Bldg. 21).

Major Findings:

1. Effect of Metal Ions on Radical Yields in γ-irradiated DNA and Related Substances (with Dr. E. Rotlevi)

The effect of certain transition metal ions (Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}) and of paramagnetic rare earth ions (Pr^{3+}, Nd^{3+}, Sm^{3+}, Eu^{3+}, Ho^{3+}, Yb^{3+}) on the formation of free radicals in γ-irradiated DNA in the solid state was investigated. Diamagnetic ions such as Zn^{2+}, Cd^{2+}, Hg^{2+}, La^{3+} and Lu^{3+} had no significant effect. Complexes of the paramagnetic ions, present in the ratio of 1 metal ion to 10 nucleotides, reduced the initial

radical yield of carbon radicals by the following ratios:

$$Fe^{2+} (\cdot\ 12) > Fe^{3+} (\cdot\ 18) > Ni^{2+} (\cdot\ 26) > Co^{2+} (\cdot\ 37) > Eu^{3+} (\cdot\ 47) >$$

$$Yb^{3+} (\cdot\ 58) > Pr^{3+} (\cdot\ 78) > Sm^{3+} (\cdot\ 83) > Ho^{3+} (\cdot\ 87) \sim Nd^{3+} \approx DNA$$

The reduction in radical yield was clearly observable when the metal ions were present in the ratio of 1 metal ion per 100 nucleotides: Fe^{2+} ($\cdot$ 52) > Ni^{3+} ($\cdot$ 60) > Fe^{3+} ($\cdot$ 77). At a ratio of 1:200, the reduction for Ni^{3+} was $\cdot$ 76.

Some other important observations regarding these metal ion effects are as follows:

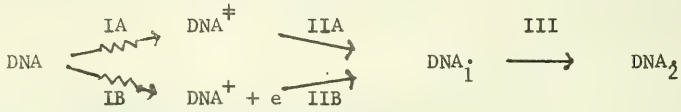
(a) The radical yields are reduced by the same amount at low doses (the linear region where the only process is radical production by ionizing radiation) and at high doses, where dose saturation occurs and the rate of radiation-induced destruction of radicals is equal to the rate of radical formation.

(b) The ions Fe^{2+}, Fe^{3+}, Ni^{2+} and Eu^{3+} have no effect on the rate of thermal decay of the carbon radicals at room temperature.

(c) There is probably little effect due to the anion of the metal salt added. The same result was obtained with Nickel chloride and sulfate.

(d) There is no significant reduction in radical yield when Ni^{2+} ions were complexed with thymine and thymidine (1:10). However, with polyadenylic acid, the effect of Ni^{2+} is approximately the same as with DNA. Single stranded DNA prepared by heating to 90° C and rapid cooling behaves like native DNA. The absence of an effect in thymine and thymidine strongly suggests that the reduction of the amplitude of the electron spin resonance signal is not the result of a spin-spin interactions between paramagnetic ions and carbon radicals.

These results can be discussed more easily in terms of the following equation:



The absorption of ionizing radiation by DNA results in the formation of excited molecules and of ions and secondary electrons. Dissociation of excited states and recombination of the ions with secondary electrons leads to the primary neutral radicals (DNA$_1$·) which are transformed to secondary stable radicals (DNA$_2$·). Radiation-induced hydrogen transfer occurs predominantly during Step III. The effects of the metal ions may take

place during Step IIA, IIB or possibly III. The relative effectiveness of different metal ions in reducing radical yield does not correlate with the magnetic moment of the ion or its second ionization potential (a measure of the electron affinity of the divalent ion). The effects observed in the current work show many interesting similarities to the quenching of the ultra-violet radiation-induced phosphorescence in DNA and polyadenylic acid by paramagnetic transition ions observed by earlier workers. These effects were observed in aqueous glasses at 77° K and have been explained in terms of quenching of triplet excitons with a diffusion length of about 100 bases. Furthermore, the relative effectiveness of the rare earth ions in reducing the free radical yields parallels the previously observed efficiency of intramolecular electron energy transfer in rare-earth chelates.

The possibility that the metal ions also act as electron and/or hole scavengers, thus interfering with Step IIB, also remains. Experiments are in progress which may help to distinguish between these possibilities. For DNA-metal complexes with a ratio of metal/nucleotide of 1:200, the average distance between metal ions along the chain is 340 Å. This would be the relevant distance for a mechanism which involves the quenching of triplet excitons travelling along the double helix. For a mechanism based on scavenging of electrons diffusing in 3 dimensions, the average separation of metal ions is 32 Å. Whichever mechanism applies, the effects of certain metal ions on radical yields at metal to nucleotide ratios of 1:200, require that the migration of excitons and/or of charge over large distances is an efficient process and plays an important role in localizing the absorbed radiation energy in these biologically important macromolecules.

2. Radiation-Induced Hydrogen Transfer in Proteins and Nucleic Acids in the Solid State

A. Ribonuclease

The mechanism for the radiation-induced hydrogen transfer from exchangeable sites (those hydrogens attached to nitrogen and oxygen) to carbon-bound sites was further investigated. For ribonuclease, it was shown that most of the hydrogen transfer takes place during the conversion of the radicals stable at 195° K to the radicals stable at room temperature. This conclusion was based on the following experimental observations. First, hydrogen-transfer was linear as a function of dose in the 0-30 Megarad region. Second, addition of sulfur hexafluoride, an excellent electron scavenger, before radiolysis had no effect on the G-value (number of hydrogen atoms transferred per 100 ev absorbed) for hydrogen transfer, but nitric oxide reduced the value to about one-tenth. Thus, the effect of nitric oxide is due to its ability to scavenge free radicals and not due to electron-scavenging. Third, irradiation at 195° K decreases the G-value for hydrogen transfer by a factor of about 6. Heat treatment at room temperature of samples irradiated at 195° K increases the hydrogen transfer three-fold. However, if nitric oxide is added after irradiation at 195° K, the increase in hydrogen transfer due to heat treatment is completely suppressed. These experiments demonstrate the dependence of

hydrogen transfer on the free radical content of the ribonuclease at 195° K and eliminate the possibility of a thermally-induced hydrogen exchange in small regions of more than average thermal energy produced by γ-radiolysis.

B. DNA

For DNA, there is less direct evidence regarding the detailed mechanism of radiation-induced transfer than for ribonuclease. However, two significant similarities have been observed between the radiation-induced hydrogen transfer in DNA and ribonuclease. First, in both substances, the G-value is reduced by a factor of about 6 when the temperature of irradiation is lowered from 298° to 195° K. Second, it was observed that for both substances, hydrogen transfer was a linear function of dose in the critical low dose region. Hence, it may be inferred that for DNA also most of the hydrogen transfer occurs during the conversion of the radicals at 195° K to the stable radicals at room temperature. It was therefore to be expected that the addition of those metal ions which decrease the radical yield at room temperature, would also decrease the G-value for hydrogen transfer. Several paramagnetic transition metals and rare earths were investigated at a metal/nucleotide ratio of 1:10. The relative order of effectiveness was:

$$Cu^{2+} > Fe^{2+} \sim Fe^{3+} > Ni^{2+} > Co^{2+} > Sm^{3+} > Cd^{2+} = DNA$$

Cadmium, a diamagnetic ion, had no effect. These results show a marked parallelism to the ESR studies of Rotlevi and Riesz on the free radical yields measured by ESR. They represent an independent confirmation of the conclusion that the reduction of amplitude in the ESR spectra of the metal-complexes is the result of a decreased carbon radical yield and is not due to the spin-spin interaction of paramagnetic ions and carbon radicals.

Significance to Biomedical Research and the Program of the Institute: Studies of the effects of ionizing radiation are of importance in relation to (1) radiation therapy, (2) carcinogenesis, (3) stability of the genetic pool, (4) the suppression of the immune mechanism, and (5) aging. The effects of ionizing radiation on nucleic acids are being studied in order to understand the nature of radiobiological death in normal and tumor cells. The addition of radioprotective and radiosensitizing agents is being investigated so that a therapeutic advantage may be gained.

National Cancer Institute research plan: Objective 6, Therapy. Approach 1, Develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (alone and in combinations) in the cure and palliation of cancer.

Proposed Course of Project:

To continue studies on the effects of ionizing radiation on macromolecules of biological importance. The mechanism of radioprotective and radiosensitizing agents will be investigated.

Honors

Invited lecture. Farkas Memorial Symposium: The Molecular Basis of Radiation Biology. The Hebrew University, Jerusalem, January, 1972.

Publications:

Smitherman, T. C., Moss, H. M., and Riesz, P.: Radiation-induced hydrogen transfer in nucleic acids, proteins and related substances: Effects of temperature and scavengers. Radiat. Res. 46: 251-267, 1971.

Miller, J. H., White, F. H., Jr., Riesz, P., and Kon, H.: Distributions of free radicals among amino acids from lyophilized ultraviolet-irradiated proteins. J. Photochem. and Photobiol. 14: 577-588, 1971.

Riesz, P., and Smitherman, T. C.: Radiation-induced hydrogen transfer in nucleic acids and proteins in the solid state. Israel J. of Chem. (accepted for publication)

ANNUAL REPORT

THE ENDOCRINE EVALUATION BRANCH AND BREAST CANCER TASK FORCE PROGRAM
July 1, 1971 - June 30, 1972

INTRODUCTION

Continuing the reconstruction of program begun the previous year, the Breast Cancer Task Force completed its outline of a "General Research Logic" for breast cancer and started implementing it. Subcommittees were appointed to concentrate on the development of various portions of the plan and have begun to promote and evaluate projects. The Endocrine Evaluation Branch has continued in a central coordinating role with respect to contracts and collaborative group activities, and is steadily reorganizing the branch structure to improve these functions.

PROGRAM AIMS AND RESOURCES

The basic aim of the Task Force--greatly improved diagnosis, prognosis and treatment through clinical and experimental research--has remained the same. However with the adoption of the expanded research plan a restructuring of advisory committees and staff became necessary, and objectives and priorities had to be revised. The internal Steering Committee of 10 members was joined with the non-governmental advisory component of 11 members to form the new Task Force. To assign responsibility for more intensive development of program areas, four new consultant groups were set up: Subcommittees on Diagnosis, Epidemiology, Experimental Biology, and Treatment. These subcommittees correspond roughly to areas of the flow chart outlining the program. All members of the Task Force were given subcommittee assignments and additional consultants were also brought in. Unlike any part of the original Task Force these subcommittees have the official function of reviewing contract proposals for scientific merit; thus they become the operational mechanisms for bringing together the scientific objectives of the program and the funding process.

The operation of a large task force program requires an internal management group concerned with such matters as the programing and writing of proposals, the mechanisms of meetings and travel, and scientific as well as organizational liaison. This has been the function of the Endocrine Evaluation Branch, which is being reorganized pari passu with changes in scientific program and committee structure, and which will be renamed in the future appropriately to the role. The reorganized branch will have a section to coordinate the work of each subcommittee, and the head of the section will be the executive secretary of the respective subcommittee.

The above-mentioned changes necessarily brought about changes in the timing and tempo of Task Force activities. The Task Force, charged with general overview of the program, now meets only once a year, for a detailed review of contract work under way. The Steering Committee, charged with maintaining scientific balance and priorities, meets approximately twice a month.

The subcommittees, which initiate, develop and review projects, meet approximately every other month. The Endocrine Evaluation Branch, charged with management and monitoring of the program, carries out its functions on a daily basis.

THE CURRENT BREAST CANCER PROGRAM

The projects currently under way fall into five categories: discriminants for clinical prognosis, tumor models for study of the biology of breast cancer, early diagnosis, preclinical drug therapy and immunotherapy.

Discriminants for Prognosis. The discriminant program aims at finding means of anticipating the course of disease at various stages and for different conditions of treatment. Discriminants that have been proposed include those based on a tumor factor (e.g., estrogen-receptor levels), and those based on host condition or host-cancer interaction, as shown by urinary steroids and patterns of clinical and histopathological data.

The attempt by Dr. Elwood Jensen of the University of Chicago to predict the response of a patient to adrenalectomy, by assaying the estrogen-receptors of biopsied tumor, appears to be close to validation. Eleven out of 14 patients with high receptor titer showed regression of disease after endocrine ablation, while only one of 32 with negative or very low readings had a favorable response. If this is confirmed, the use of this test would make it possible to eliminate most of the surgical ablations for advanced breast cancer that would fail to help the patient.

Patients undergoing mastectomy for primary breast cancer may have a "free period" lasting a few months up to several years. If the physician knew his patient was in the high risk category at this time he would have good reason for instituting additional therapy at once instead of waiting for progression. The approach taken in the Primary Breast Cancer Study was to determine whether the high risk patient could be distinguished through the use of a discriminant based on urinary steroids and the tissue pathology of the tumor and lymph nodes. Clinicians of three institutions--Alton Ochsner Clinic, Yale University and the University of Pennsylvania--furnish the urine specimens and tissue samples from their patients in the study. The tissues are processed at the hospitals of origin and sent to the St. Vincent Hospital (Worcester) for confirmation and study, and the urine specimens are sent to the Worcester Foundation for Experimental Biology for steroid profiles. Documentation and statistical work for the project are carried out at Louisiana State University. Scheduling of services is kept in balance by the LSU Statistical Center and the Endocrine Evaluation Branch. A supplementary project has been started at Lackland Air Base to include women with non-cancerous lesions of the breast and other controls. Contracts with the State University of New York, Henry Ford Hospital and the University of Vermont College of Medicine are being negotiated, to increase and complete patient input by the end of 1972.

A second contract facility for automated steroid analysis was set up at the Research Foundation for Mental Hygiene in Orangeburg, N. Y. This made it possible to extend the discriminant approach to three other projects. One

was organized to determine the validity of the Bulbrook discriminant, or another steroid-based discriminant, as a predictor of success for hypophysectomy or adrenalectomy in advanced breast cancer. The second, an additional control for the primary Breast Cancer Study, is determining the urinary steroid pattern in endometrial cancer. The third project is based in Israel, where endocrine and anthropometric studies are being carried out on three ethnic groups--Yemenites, who have a low incidence of breast cancer, other Asians who have 3.5 times as much, and Israelis of East European extraction who have 6.5 times as much. Urinary specimens from these groups are sent to the Orangeburg facility for steroid analysis.

Finally, it would be desirable to recognize when a regression is under way, since detectable response to treatment is not always rapid or dramatic. One attempt is being made, at Jefferson Medical College, to find an early indication of response--in this instance, to see whether changes in the emission of heat from a tumor can be used to indicate responsiveness to a drug, in a matter of days rather than weeks. The pattern of heat change in the area of a breast tumor sometimes does change when hormones are administered, but data are not yet sufficient to show whether or not these changes are related to tumor regression.

Tumor Models for Research and Testing. Model tumor systems are essential for studies on the biology of breast cancer as well as for the testing of drugs. The Endocrine Evaluation Branch has investigated many neoplasms of mammary tissue in its contract projects and utilizes some of them regularly for research and testing. Since most of the useful endocrine-related tumor systems are found in inbred rats, a tumor bank and host-genetics monitoring facility was set up at the Mason Research Institute in Worcester. This supplements the facilities set up by Chemotherapy for monitoring mouse stocks and their tumors. The tumor bank contains specimens of 51 tumors, many of them now unobtainable from other sources.

DMBA-induced mammary tumors in Sprague-Dawley rats serve as a model for primary breast cancer and consequently are much studied. Investigators at the Southern Research Institute are studying the cell kinetics of such tumors as affected by ablative surgery and drug therapy. A DMBA-induced tumor in the Fischer/344 rat, the 13762 mammary adenocarcinoma, has been used as a transplantable model system for a variety of purposes at the Mason Research Institute; it has been used routinely there for testing new compounds and is currently being used to study the effects of surgery, chemotherapy and immunotherapy on metastases that appear after removal of the primary graft. It was found last year that this tumor has an anti-uterotrophic effect, apparently by depriving the uterus of endogenous estrogens--an effect for which there is also some evidence in patients with breast cancer.

Another model system used at Mason Research Institute, the R-35 mammary adenocarcinoma of the Sprague-Dawley rat, was found to contain a "C-type" virus. This was one result of collaboration between this Branch and the Special Virus Cancer Program (Etiology) to screen all tumors available at EEB contract facilities for viruses and to seek viruses in human breast tumors and milk. Three of the contract facilities used for these purposes

by the Special Virus Cancer Program--at Georgetown University, Institute for Medical Research, and Charles Pfizer & Company--have been supported in whole or in part by Breast Cancer Task Force funds.

Early Diagnosis. The Breast Cancer Task Force has a Pathology Working Group which has the problem of early breast cancer lesions under constant review. In the contract program two projects have been supported at the University of California, where the "hyperplastic alveolar nodules" of DeOme became known as the outstanding animal model of early lesions. The projects aim at utilizing new techniques to study sub-gross and microscopic lesions in the breasts of dogs and human beings, to establish norms of preneoplastic manifestations in these species. An "Atlas of Subgross Pathology of the Human Breast" is being prepared, based on studies of 295 specimens from accident victims or routine autopsies. In addition to these a project for the study of the ultrastructure of borderline lesions has recently been approved for funding, and another for a statistical study of early lesions of the breast is being considered. The possibility of utilizing immunological methods for the early diagnosis of breast cancer has been extensively discussed by the Diagnosis Subcommittee and has been placed in the highest priority category.

Preclinical Drug Therapy. Due to a policy decision, activities of the Branch having to do with procurement and screening of endocrine compounds for clinical use have been phased out. The contracts for synthesis of steroidal antitumor agents (Research Triangle Institute), large scale resynthesis of steroid derivatives (Upjohn) and quality control of hormonal agents (University of Tennessee) are on a terminal year.

Immunotherapy. Two years ago a contract was let with Hebrew University (Hadassah Medical School, Jerusalem) for "Preclinical Studies on a Tumor-Protective Activity of a Methanol Insoluble Fraction of Attenuated Tubercle Bacilli (BCG)." The immediate objective of this project is to develop appropriate animal models for the study of adjuvants to immunological protection against breast cancer. Dr. Weiss is using mice in this contract; Dr. Bogden at Mason Research Institute is carrying out studies with rats and a methanol-soluble fraction of M. butyricum and H. pertussis. As a more specific step in the direction of immunotherapy, it has been proposed by Dr. Olof Pearson to develop a vaccine containing antibodies to human prolactin, since animal experiments indicate that prolactin is a potent stimulator of breast tumors.

The Current Program: Research Priorities

During the last quarter of 1971 the Steering Committee and the subcommittees worked intensively on priorities within the various areas of the charted research program. Each subcommittee decided upon as many as five to fifteen areas within their respective sectors of the Flow Chart, to be deserving of high priority. The Steering Committee further narrowed these to a smaller number for immediate attention:

Diagnosis: (1) a comparative study of thermography, clinical examination and some variant of Molydenum mammography--xerography; and

(2) immunodiagnosis

Epidemiology: (1) methodology and technology of determining levels of estrogens and other steroids in blood;

(2) development of specifications for a study of cystic mastopathy in relation to breast cancer; and

(3) genetic studies

Experimental Biology: (1) growth regulation of mammary tissue,

(2) hormonal requirements,

(3) role of immunological mechanisms in tumor development and regression;

(4) biological events related to metastasis; and

(5) viral events in the etiology and progression of mammary tumors

Treatment: (1) surgery plus systemic treatment--radiation, immunotherapy, chemotherapy;

(2) biopsy of the opposite breast

At the time of this writing some 20 contract proposals, most of them new, are under evaluation for funding with FY-1972 money. A number of these are aimed to fulfill the priorities listed above in the field of Experimental Biology. In the clinical field a contract with the University of Pittsburgh has been approved for a large collaborative study consisting of two parts: one, a comparison of simple ("total") and radical mastectomy; the second, a study of prolonged chemotherapy as an adjuvant to mastectomy for primary breast cancer.

In order to stimulate new projects all the subcommittees have devoted parts of their meetings to scientific sessions, at which investigators doing interesting work present their data. Some subcommittees also plan working conferences to clarify important problems. Thus, the Treatment Subcommittee held a two-day review of therapeutic modalities in breast cancer, attended by approximately 50 investigators (January 31 and February 1, 1972); and the Epidemiology Subcommittee is holding a two-day meeting (June 26, 27) on Hormones and Breast Cancer which is expected to produce specific plans for new epidemiological studies.

Relation to National Cancer Program Research Strategy

The Breast Cancer Task Force program is essentially the application of the National Program Research Strategy to a single organ site. The main goal is to reduce morbidity and mortality in humans--due to breast cancer. The four principal approaches of the National Program are all represented explicitly in the General Research Logic of the Breast Cancer Program. Finally, due to the complicated and variable natural history of breast cancer it is likely that few project areas of the National Program will be without some counterpart in the Breast Cancer Program.

SUMMARY OF PROGRESS AND FORECAST OF DIRECTION

The "General Research Logic for a Breast Cancer Program" was completed at the beginning of the fiscal year and served as a guide for subsequent activities. The Task Force itself was strengthened by the appointment of four working subcommittees to cover the principal divisions of the plan, and the Endocrine Evaluation Branch is being reorganized for coverage of similar divisions. By the end of this year the contract program will have doubled in size, about half the new contracts being in the area of Experimental Biology, the rest largely in Treatment and Diagnosis. Working conferences are being held to probe areas of presumptive importance. It is expected that further expansion will occur in the coming year, and that particular attention will be given to areas where specific projects have been difficult to start.

Contract PH43-64-46 - Hospital for Joint Diseases

1. Biswas, S., and Hollander, V. P.: Metabolic studies on mammary tumor MTW9 following resection of the mammosomatotropic tumor MtTW5. Cancer Res 31: 1360-1363, 1971.

Contract PH43-65-6 - Mason Research Institute

1. Rosenkrantz, H., and Chin, T.: Kinetics of hydrolysis of orthophosphate monoesters by canine prostatic acid phosphohydrolase. Invest Urol 8: 215-223, 1970.

2. Molinari, P. F., Menninger, F. F., Jr., and Rosenkrantz, H.: Detection of an inhibitor of hemoglobin synthesis in rat plasma. J Lab Clin Med 76: 466-471, 1970.

3. Molinari, P. F., and Rosenkrantz, H.: Erythropoietic activity and androgenic implications of 29 testosterone derivatives in orchiectomized rats. J Lab Clin Med 78: 399-410, 1971.

Contract SA-43-ph-2395 and PH-43-65-1050 - E. R. Squibb and Sons

1. Hilf, R., Bell, C., Goldenberg, H., and Michel, I.: Effect of Fluphenazine HCl on R3230AC mammary carcinoma and mammary glands of the rat. Cancer Res 31: 1111-1117, 1971.

Contract NIH69-33 - University of California at Davis

1. Cameron, A. M., and Faulkin, L. J., Jr.: Hyperplastic and inflammatory nodules in the canine mammary gland. JNCI 47: 1277-1287, 1971.

Serial No. NCI-15-000
1. NCI
2. GL&C
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Part A

Project Title: Screening

Principal Investigator: Dr. Alfred S. Ketcham

Other Investigators: Physicians participating in patient care

Cooperating Units: Dermatology Branch, Immunology Branch, Medicine Branch, Metabolism Branch, Radiation Branch, and Surgery Branch

Man Years:
Total:
Professional:
Other:

Project Description: This is a mechanism by which this necessary hospital function can be classified.

Part B included Yes___ No X

Serial No. NCI-15-001
1. NCI
2. GL&C
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Part A

Project Title: Screening

Principal Investigator: Dr. Alfred S. Ketcham

Other Investigators: Physicians participating in patient care

Cooperating Units: Dermatology Branch, Immunology Branch, Medicine Branch, Metabolism Branch, Radiation Branch, and Surgery Branch

Man Years
Total:
Professional:
Other:

Project Description: This is a mechanism by which this necessary hospital function can be classified.

Part B included Yes___ No X

Serial No. NCI-15-002
1. NCI
2. GL&C
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Part A

Project Title: Emergency Admissions

Principal Investigator: Dr. Alfred S. Ketcham

Other Investigators: Physicians participating in patient care

Cooperating Units: Dermatology Branch, Immunology Branch, Medicine Branch, Metabolism Branch, Radiation Branch, and Surgery Branch

Man Years
Total:
Professional:
Other:

Project Description: This is a mechanism by which this necessary hospital function can be classified.

Part B included Yes___ No X

SUMMARY STATEMENT
ANNUAL REPORT
Dermatology Branch - NCI
July 1, 1971 through June 30, 1972

Dermatology Staff:

Senior Investigators:

Marvin A. Lutzner, M.D. (Branch Chief)
William R. Levis, M.D.
Gary L. Peck, M.D.
Jay H. Robbins, M.D.
Arthur R. Ugel, M.D.
Bruce K. Wetzel, Ph.D.

Staff Fellow:

Vincent J. Hearing, Jr., Ph.D.

Clinical Associates:

Thomas P. Nigra, M.D. - 3rd year
Stephen B. Guss, M.D. - 2nd year
Douglas J. Key, M.D. - 2nd year
Kenneth H. Kraemer, M.D. - 1st year
A. Edgar Miller, Jr., M.D. - 1st year

The Dermatology Branch studies the growth and differentiation of epithelium and lymphoreticular tissues in normal hyperplastic and neoplastic states. The major thrust of the Branch effort is to study the skin from the point of view of both its basic biology and its clinical diseases. In addition to its research efforts, the Branch serves as the dermatologic consultant to the Clinical Center.

For the past several years the major clinical and laboratory interests of the Dermatology Branch have been: (1) the development of therapy for cutaneous malignancies and benign hyperproliferative dermatoses, (2) elucidation of the gene defects in benign and precancerous inherited diseases of the skin (genodermatoses), (3) the ultrastructure of normal and diseased skin, (4) the immunologic role of the lymphocyte, (5) the biochemistry and tissue culture of normal and diseased skin, and (6) study of the pathogenesis of specific skin diseases.

1. The Development of Therapy for Cutaneous Malignancies and Benign Hyperproliferative Disorders:

Topical CCNU in the Treatment of Psoriasis. Nine of twelve patients treated in a double-blind manner with topical 1.0% CCNU, 1-(2-Chloroethyl)-3-cyclohexyl-1-nitroso-urea, had resolution of psoriatic plaques in 4-6

weeks. Local toxicity included cutaneous irritation with epidermal separation, hyperpigmentation, telangiectasia, and cutaneous pain and pruritus. Two patients developed temporary bone marrow suppression. Four of the twelve patients have received 18 month remissions to date.

Topical BCNU and CCNU in the Treatment of Basal Cell Epithelioma. Eleven of twenty-four lesions of basal cell epithelioma treated with 0.1% BCNU disappeared completely, both clinically and histologically. Ten other lesions showed partial regression. No visible effect was seen on three lesions. No recurrences have been noted to date. The longest follow-up period is over twenty-four months. The results with CCNU were less impressive. Only one of seven lesions treated with 0.4% CCNU showed complete regression. Four of seven responded partially.

Xeroderma Pigmentosum. An unexpected beneficial effect was observed in a split-thickness skin graft site in a patient with xeroderma pigmentosum. The number of pigmented lesions, keratoses, and skin tumors was remarkably reduced as compared to the surrounding skin. Subsequently, several other severely diseased areas were treated by dermatome stripping with uniformly beneficial results.

Effect of Delayed Hypersensitivity Reactions on Basal Cell Carcinomas. Currently, studies are aimed at assessing the efficacy of 2,4 Dinitrobenzene (DNCB) applications to tumors in sensitized individuals. A 95% success rate using topical DNCB in premalignant keratoses, superficial basal cell carcinomas and squamous cell carcinomas in situ has been reported. We are attempting to confirm this report and to evaluate the effect of different concentrations of DNCB, different schedules, different vehicles, and the effect of DNCB on the various types of basal cell carcinomas (e.g. superficial, pigmented, nodular-ulcerative, sclerosing). When these parameters are worked out and if the efficacy of DNCB therapy of basal cell carcinomas is significant in our hands, additional studies on the role of delayed hypersensitivity in this process will be pursued.

Preliminary results, to date indicate that DNCB has some effect in the treatment of basal cell carcinomas. Three patients had an over all decrease in tumor mass in many treated lesions. Only a small number of the total tumors treated disappeared completely with the longest follow up being 6 months.

Topical Chemotherapy Screening Proposal. Efforts at establishing a national cooperative group for the screening of new chemotherapeutic drugs topically in the treatment of psoriasis, basal cell carcinoma, and mycosis fungoides have been made during the past year. Drugs will be screened for efficacy in preclinical systems. Active drugs will then be screened for toxicity. Safe, active drugs will then be formulated and distributed to leading dermatologic training centers for evaluation.

Topical Vitamin A in the Treatment of Psoriasis. No preferential beneficial response was seen in the treatment of 14 psoriatic patients using Vitamin A Acid cream without plastic occlusion in concentrations of 0.05% to 0.3%.

Three of nine patients treated with 0.25% Vitamin A Acid cream under plastic occlusion in a double blind manner showed definite evidence of clearing limited to Vitamin A Acid treated areas. This response, which was first evident 2 to 4 weeks after onset of therapy, was short-lived in two of these patients, who also failed to respond to subsequent courses of therapy.

2. Elucidation of the Gene Defects in Benign and Precancerous Inherited Diseases of the Skin (Genodermatoses):

Darier's Disease. Accelerated epidermal wound healing was found to occur in one patient with Darier's Disease. Superficial linear incisions healed by 12 hours in diseased skin, and by 48 hours in uninvolved skin. Dermatome stripped diseased sites healed faster than disease-free stripped sites. Additional clinical observations were that dermatome stripped disease-free skin was resistant to disease during subsequent generalized exacerbations, and that dermatome stripped diseased skin not only healed disease-free, but that the surrounding 5-10 centimeters of diseased skin also healed simultaneously. This last result suggests that some beneficial, diffusible substance may be present in the wound site.

Additional studies will be done in order to confirm accelerated epidermal wound healing in this patient. This process is also being studied ultrastructurally.

Epidermolysis Bullosa Simplex. Epidermolysis bullosa simplex is an autosomal dominant blistering disease caused by traumatic disruption of epidermal basal cells. One hypothesis for the pathogenesis of this disease is that lysosomal activity was increased in this disease. Accordingly, lysosome activity in epidermolysis bullosa simplex was studied using the electron microscope. Acid phosphatase, a marker enzyme for lysosomes has been studied in this disease and found to be normal. An unexpected finding occurred in the study: i.e., the observation that there is apparently a nonspecific attraction of the melanin granule for lead nitrate used in the standard histochemical test for acid phosphatase. This observation opens questions concerning findings by others using this method in studying acid phosphatase activity in melanin granules.

Porokeratosis Plantaris, Palmaris, et Disseminata. Eight members from four generations of a family had multiple superficial porokeratoses on the trunk, palms, and soles. This appears to be a third type of porokeratosis, distinct from the classic Mibelli type or the Chernosky disseminated supercial actinic porokeratosis (DSAP) type. The disease begins in the late teens and early twenties, is transmitted as an autosomal dominant, and affects males twice as often as females. The hallmark of this disease is the early appearance of plantar and palmar lesions with subsequent involvment of other areas of the body including surfaces not exposed to ultraviolet radiation. Morphologically, the clinical lesions resemble Chernosky's DSAP. This third type of porokeratosis may be a new precancerous genodermatosis as one of our eight patients developed several squamous cell carcinomas in lesions of porokeratosis.

Ehlers-Danlos Syndrome. A family with two members having the typical

clinical appearance of Ehlers-Danlos Syndrome has been found to have hydroxylysine deficient dermal collagen. Peptidyl lysine hydroxylase deficiency is the presumptive defect in this hereditary disorder of connective tissue.

Xeroderma Pigmentosum. Xeroderma pigmentosum (XDP) is a genodermatosis with a marked predisposition to development of carcinomas in sun exposed areas. A defect in DNA repair, post UV-damage, has to date, been shown in all but three of these patients.

The prolonged UV-induced ^{3}HTdR incorporation in XDP lymphocytes has now been shown to occur in UV-irradiated cells of each of our 7 XDP patients who have an abnormally low rate of incorporation immediately after irradiation. Our data suggest that these XDP cells can perform as much DNA repair as normal cells but require a longer period in which to accomplish this incorporation. Studies of UV-induced ^{3}HTdR incorporation into normal and XDP epidermal cells are being conducted. The preliminary results indicate that the results with epidermal cells correlate with results from fibroblasts and lymphocytes. In view of the 3 known XDP patients with normal UV-induced, DNA repair, it is likely that the malignancies and clinical signs of XDP may have more than one etiology or that the repair defect present in the great majority of XDP patients may be etiologically unrelated to their disease.

The question arises as to whether the carcinogenesis shown in these patients is perhaps the expression of oncogenic virus activity initiated or allowed by the suspected enzyme defect in UV-DNA repair in these patients. Accordingly, the susceptibility of both normal and XDP skin fibroblasts to transformation by oncogenic viruses after UV-irradiation was studied. However, with SV-40 virus infection post-irradiation there was no enhancement, but a decrease in transformation in proportion to the post-irradiation survival curve. The T antigen incidence, as a rough marker of infectivity, was the same in irradiated as non-irradiated cells as measured 48 hours post infection. These studies are now being continued with other viruses.

3. The Ultrastructure of Normal and Diseased Skin:

The Fine Structure of Leukocyte Granules. The development, function, and fate of granular leukocytes and mast cells and the origin, nature and fate of their characteristic cytoplasmic granules are being studied in bone marrow, peripheral blood, tissue culture, and in the dermis where they congregate in certain diseases. Major emphasis is placed on quantitative ultrastructural autoradiography to elucidate patterns of granulogenesis and function.

A comparative study has been done of the effects of various fixatives on developing granulocytes in rabbit marrow has resolved a serious discrepency in the literature in which early stages in neutrophil development have been interpreted as a special form of early eosinophil; eosinophil granules are much more resistant to extraction and other artifacts than neutrophil granules, and can thus be readily distinguished in specimens where the neutrophils are consistently altered by the fixative.

Scanning Electron Microscopy. An effective and reliable method which

employs glutaraldehyde fixation and critical point drying with CO has been chosen and modified for the preparation of soft biological tissues for SEM. With this approach, surface changes in various systems have been studied, such as intestinal explants in organ culture, cultured leukocytes from human buffy coat, lymphocyte blastogenesis, rat thyroid follicles after exposure to thyrotropin, and chick embryo skin after exposure to Vitamin A Acid in organ culture.

Biosynthesis of Melanin in Normal and Malignant Cells. This research involves the structural composition of the various types of melanin granules, both in normal and abnormal cells. A series of pigment mutants of the mouse has been investigated thus far which are analogues of normal and abnormal human pigmentation: black (normal), white (albinism) and beige (Chediak-Higashi syndrome), among others. The results of this phase of our work indicate that a common pathway of melanogenesis exists, which is common to all pigment mutants studied. A fibrillar premelanosome is formed, upon which melanin is uniformly deposited. Eventually the melanized fibers become so thick that fusion between them apparently takes place, and all structural detail is masked. In the mutants studied, the basic difference from normal melanogenesis as described above is the premature termination of melanin synthesis at one of several points. This results in incompletely formed, rather than aberrant, granules. One exception to this is found in the beige animal, in which many normally forming granules fuse together, resulting in giant granules. Since the fusion of other types of granules in these beige animals in noted, the mechanism of fusion is not restricted to pigment granules alone.

Graft-Versus-Host Disease. The first reported case of Toxic Epidermal Necrolysis developing in a patient with Graft-Versus-Host Disease following bone marrow transplantations has been studied with the electron microscope. Electron microscopic findings in biopsies taken at distances greater than 10 cm. from the blister edge, ultrastructural changes were limited to the basal cell layer. The entire basal layer was involved in a uniform manner, while the spinous, granular, and horny layers appear virtually uninvolved. Mitochondrial swelling, coarsening of tonofibrils, and increased prominence of ribosomes was noted in all basal cells. The nuclei appeared contracted with more convoluted nuclear membranes; increased density of chromatin was noted. Biopsies closer to the blister edge displayed considerable intercellular edema, and occasional early bulla formation. Lymphocytes were occasionally seen within the basal layer, but only rarely above. Increased numbers of Odland bodies and abnormal keratohyalin granules were noted in the superficial epidermis. In summary, the initial pathologic events are in the basal layer, and it appears that the mononuclear infiltrate may play a significant pathogenetic role.

Vitamin A - Induced Mucus Metaplasia as a Model for Epidermal Differentiation. The effect of Vitamin A Acid on the induction of mucous metaplasia in 14-day chick metatarsal skin was studied in organ culture using the electron microscope. It was found that whereas control specimens keratinize normally with the formation of thick bundles of tonofilaments, keratohyalin granules and a multi-layered stratum corneum, Vitamin A Acid treated specimens did not undergo keratinization. Stratum corneum and keratohyalin

granules were not formed, and mucous droplets were present in the cytoplasm. Glandular acini were also observed within the epidermis. This process has also been examined by the scanning electron microscope revealing an irregular array of long microvilli on the rounded, metaplastic, epithelial cells.

Mouse Model of Ritter's Disease. The electron microscope has been used to examine the mouse model of Ritter's disease, an exfoliative skin disease of children. It has been found that this disease is caused by a Group II staphylococcal exotoxin. In newborn mice this exotoxin has been shown to produce exfoliation of the skin of these mice similar to that which occurs in children. We have been studying the early ultrastructural effects of this exotoxin which apparently causes separation, exclusively, of cells within the granular layer. Five minutes following injection there appears a peculiar segmentation of one desmosomal line, followed by splitting of the desmosome into halves and separation of the cells from one another.

In Vitro Carcinogenesis. Mouse epidermal cells were transformed in vitro by the carcinogen dimethylbenzanthracene. These transformed cells were then injected subcutaneously into isologous mice. Resulting squamous cell carcinomas were biopsied and resuspended in culture. At all stages these cells and tissues were monitored by electron microscopy in an attempt to establish the nature and origin of the cells undergoing malignant transformation. By identifying tonofibrils and desmosomes, the transformed cells have been identified as being epidermoid in nature.

Mycosis Fungoides and Sezary's Syndrome. With the electron microscope cells with bizarre indented, lobulated, and serpentine nuclei have been found in skin, lymph nodes, and blood from patients with mycosis fungoides and Sezary's syndrome. Patients with parapsoriasis en plaque, a disease which often progresses to mycosis fungoides, also have this atypical cell. Thus, recognition of these cells should aid in the early diagnosis of mycosis fungoides. However, this cell is not specific for M.F. or for malignant disease, as it has been found in benign disease.

Recent evidence showing that the Sezary cell undergoes mitosis in vitro only with addition of phytohemagglutinin and that Sezary cells are devoid of immunoglobulins at their cell surface may be interpreted as indicating that the Sezary cell is a thymus-derived lymphocyte, and that the Sezary Syndrome may represent a malignancy of this cell type.

Osmium Zinc Iodide Stain for the Golgi Apparatus. Osmium zinc iodide has been used as a special stain for the Golgi apparatus and other subcellular organelles in the Hydra. Four hours after combined fixation-staining with O_8O_4 - ZnI_2 (OZI), the stain was limited to the Golgi apparatus and certain surface coats of Hydra littoralis and Hydra pseudoligactis. Adjustment of the staining solution to neutral pH with .1N NaOH results in staining limited to the Golgi apparatus with remarkable uniformity, clarity and consistency. Shorter or longer staining with OZI produces only partial Golgi staining and absence of surface coat staining. The luminal surfaces of large intracellular vacuoles were also delineated under certain conditions, and staining in some sites is abolished by pretreatment with sialidase or hyaluronidase, suggesting the involvement of acid mucosaccharides in sites, which

stain with OZI. Elongated coated vesicles of Hydra epithelium, reported analogous with the enigmatic Langerhans granules of human epidermal Langerhans cells, have failed to stain with OZI, thus establishing a difference between these organelles.

4. The Immunologic Role of the Lymphocyte:

Tissue Culture Studies of Human Lymphocytes. The lymphocyte is a cell involved in immune reactions which may be operative in the immune response to neoplastic tissue. Our studies concerned with blastogenesis and the control of cell division and differentiation of the lymphocyte may elucidate this cell's normal and abnormal functions in the immune response involved in the host's defense against cancer.

Methods for obtaining 'purified' lymphocytes, glass-adherent mononuclear cells, and a population containing both cell types from human peripheral blood have been developed and analyzed. We have improved and utilized a very rapid and simple 5-minute centrifugation method using heparinized whole blood for obtaining a population of mononuclear leukocytes (lymphocytes and monocytes) with very few contaminating granulocytes. This mononuclear leukocyte population contains the two cell types required for the blastogenic response and provides cultures capable of responding with maximal responses to specific and non-specific blastogenic factors. Parameters affecting the millipore filter assay technique for measuring tritiated thymidine incorporation into DNA in leukocyte cultures have been studied and analyzed. The applications and limitations of the assay method have been determined, and it has been successfully utilized in detecting DNA synthesis in microcultures containing less than 5×10^4 leukocytes.

The performance of a specifically designed autoclave has been evaluated after its initial years of operation. This autoclave has a unique stainless steel steam generator which permits the use of distilled water to generate steam virtually free of organic or ionic contaminents which might otherwise be deposited on tissue culture glassware or utensils during sterilization. The recognized advantages of this autoclave have led to its use in several other tissue culture laboratories at the National Institutes of Health and elsewhere.

Morphological studies of cellular interaction among mononuclear cells in leukocyte cultures have resulted in an experimental model and mathematical analysis of cell cluster formation. It is now possible to detect, measure and analyze such cluster formation not only in PHA-stimulated cultures but also in antigen-stimulated cultures as early as 2 hours after the start of incubation. Functional studies of supernates from unstimulate macrophage and leukocyte cultures have revealed the presence of a soluble factor(s) which can restore blastogenic responses to 'purified' lymphocytes stimulated either with antigens or allogeneic 'purified' lymphocytes in the absence of added macrophages. Supernates from PHA-stimulated leukocyte cultures have been studied for their ability to inhibit DNA synthesis in leukocyte cultures. These biologically active factors are to be characterized, purified and isolated.

The technique for removing fixed and embedded cells from glass coverslips provided the means for obtaining the first electron microscopic views of unaltered intercellular contacts between lymphocytes and macrophages in human peripheral blood leukocyte cultures exposed to specific antigens or to phytohemagglutinin. Three-dimensional reconstruction of electron micrographs of these cultures revealed previously unrecognized structures termed intercellular lacunae". These sequestered microenvironments, by virtue of their ability to maintain higher concentrations of substances released by cells than can be achieved in the large pool of extra-cellular fluid, may be important components of an intercellular regulatory system.

Epithelial - Leukocyte Interactions. Human epidermal cells induced blastogenesis in allogeneic leukocytes, but not in autologous leukocytes nor in the leukocytes of a mixed leukocyte HL-A identical sibling pair. These results indicate that epidermal induced leukocyte blastogenesis is a measure of histocompatibility in humans comparable to a one - way mixed leukocyte reaction and that mixed skin-leukocyte cultures offer a useful in vitro model for study of homograft rejection and for in vitro studies of contact dermatitis.

Use of Methanol Fixed Paraffin Embedded Epidermis for Detection of Antinuclear Antibodies. Use of paraffin embedded cow hoof allowed a sensitivity equal to that of cryostat sections for detecting A.N.A. Furthermore, sections had a better preservation of tissue architecture, resulting in easier interpretation. This technique should facilitate the immunofluorescent detection of most, if not all, circulating antibodies, including tumor-specific antibodies.

5. The Biochemistry and Tissue Culture of Normal and Diseased Skin:

Isolation and Characterization of Keratohyalin Granules. Keratin is the chief metabolic product of the epidermal cell or keratinocyte. Within the granular layer of the epidermis, epidermal cells contain large amounts of a peculiar amorphous structure known as the keratohyalin granule (KHG). The chemistry and function of the KHG has never been elucidated completely. In our laboratory, we have developed a method for extracting and reaggregating keratohyalin granules in vitro. It has been found that pure stratum granulosum can be separated from calf hoof epidermis and can be used as a source of keratohyalin granules. These granules can be selectively extracted in a phosphate buffer 0.5M to 1.3M, pH 7.0. When the salt concentration is reduced by dialysis, a precipitate forms which when monitored with the electron microscope is composed of particles which resemble keratohyalin granules. The electron microscopy of fetal calf hoof KHG demonstrated large numbers of polysomes surrounding the granules, as well as pockets of polyribosomes within the granules. Aggregated KHG has been fractionated on disc gel electrophoresis revealing an oligomeric series of protein containing thirteen members. This oligomer has been purified and found to have a unique amino acid composition, namely large amounts of serine, histidine, arginine and glycine. Depolymerization properties of the 13 member oligomeric series has also been investigated, and a way to break all oligomers into the lowest subunit has been found. Proof of homology in this series has been demonstrated through physico-chemical studies, immunology, and kinetic studies. The latter studies

have been performed on short term tissue culture of whole hoof epidermis pulsed with radioactive precursors. The studies show that sequential labeling of the oligomeric series occurs: that is, the label moves with time from the low to high molecular weight species. Autoradiographic studies show that keratohyalin synthesis begins with 5 minutes of a radioactive pulse entering a cell.

Immunofluorescent studies prove that the isolated protein comes from the keratohyalin granules since fluorescence is specifically located over KHG. The antibody to keratohyalin reacts with trichohyalin, indicating that chemical similarities exist between the two substances. Trichohyalin granules are found in the outer root sheath of hair follicles. Immunologic studies demonstrate the antigenic similarity of bovine, ovine, wildebeest, and goat keratohyalin whereas there is no cross reactivity with human, rodent, horse, or carnivore keratohyalin. However, the keratohyalin of goat, sheep, cow, and wildebeest are not chemically identical since they have been shown to have antigenic sites which are unique to each species. Keratohyalin has also been isolated and characterized physico-chemically and histochemically from goat, sheep, and wildebeest.

The function of KHG remains unknown. In the past it has been considered to be important in forming the final keratin product in the epidermis. Since we have found that keratohyalin is composed of a basic protein complexed with RNA, an alternate hypothesis is that the bound RNA may control epidermal cell growth and development, and that the basic protein specifically binds nucleotides. This hypothesis might explain rapid growth phenomena in epidermis lacking keratohyalin, such as psoriasis, basal cell and squamous cell carcinoma.

Epidermal Differentiation. In a histochemical, electron microscopic study of chick embryo metatarsal skin in organ culture, it was confirmed that Vitamin A Acid induces epidermal mucus metaplasia. In addition, it was found that the epidermis is capable of mucus glandular metaplasia as well. This process was inhibited by the addition of puromycin or cycloheximide to the culture medium. Actinomycin D, hydroxyurea, and colchicine failed to inhibit this Vitamin A-induced process. Thus, protein synthesis is necessary for metaplasia, while mitosis, and DNA and RNA synthesis are not.

Collagen Chemistry in Normal and Disease States. Collagenase is the major degradative enzyme found to date for the collagen molecule. This enzyme has been found in a number of tissues. Heretofore, no one has identified this particular neutral degradative enzyme in human diploid fibroblast in culture. But we have isolated this enzyme in the culture system and are currently further characterizing its nature.

In fibroblast culture the various factors affecting the rate of collagen synthesis are being examined. Particularly at this time the effect of pH on precursors of collagen biosynthesis and on the enzymes of collagen biosynthesis is being examined. Recent data indicate that there is a 2-fold increase in protein synthesis and a 4-fold increase in collagen synthesis at pH 7.8 as compared to pH 7.4.

The effects of acute and chronic inflammation on collagen synthesis and degradation are being studied. In the Arthus reaction, for instance, there is a peak of collagenase activity 12 hours after initiating the lesion. Immune complexes are present; polymorphonuclear cells are maximum at this time. Furthermore, it has been shown that PMN cells in vitro release collagenase in the presence of complement by immune complexes.

In cooperation with NIDR the disease Osteogenesis Imperfecta, an autosomal dominant disease characterized by fragile bone tissue and blue sclera is being studied. When fibroblasts from patients with Osteogenesis Imperfecta are cultured, they show a defect in the amount of collagen synthesized. Also the effect of pH on the precursors of collagen biosynthesis in vitro and on the enzymes of collagen biosynthesis is being examined.

Melanogenesis in Normal and Abnormal Mammalian Tissues. The structural composition of melanin granules and the control mechanisms involved in melanogenesis are being studied. Preliminary results indicate that the normal melanin granule is composed of only five proteins, four of which are loosely bound (most likely forming the limiting membrane), the other being tightly bound within the melanin polymer matrix (presumably the fibrillar structural protein).

Since only one enzyme (tyrosinase) is essential for melanin biosynthesis, it is a unique system for the study of enzymatic control mechanisms. Tyrosinase activity has been studied in black and albino mice. Interestingly, albinos do produce some tyrosinase, but its activity is somehow blocked in vivo. One can demonstrate this activity in vitro by treatment of the tissue with detergent (Triton X-100) or with RNase, but not, significantly, with any proteases. When one treats pigmented tissues with RNase, a slight increase in activity is observed, while treatment with Triton X-100 inhibits tyrosinase activity by approximately 80%. Proteases on the other hand, result in a large increase in activity. These results indicate inhibition control systems for tyrosinase activity in black and albino animals differ greatly, and in addition, that much work is necessary to properly evaluate them. It is anticipated that melanogenesis will be similarly studied in normal and pathological human conditions.

6. Study of the Pathogenesis of Specific Skin Diseases:

Mycosis Fungoides. One hundred and forty-four patients with mycosis fungoides were studied at the NIH between 1954 and 1968. Fifty percent of these patients died within 3.5 years after biopsy diagnosis.

Patients under 51 years of age and those without cutaneous tumors, cutaneous ulcers, or palpable lymph node enlargement survived longer after diagnosis. The development of any of these three clinical signs or of lymph node lymphoma, hepatomegaly, or splenomegaly heralded a shortened survival. Eighty percent of the 35 patients initially seen at the NIH without cutaneous tumors, cutaneous ulcers, or palpable lymph node enlargement either developed progressive disease or died of unknown or unrelated causes by January 1971.

Although irradiation and topical and systemic medications often caused good

clinical improvement, no effect of therapy on survival could be demonstrated.

At autopsy, widespread visceral lymphoma was frequently found, and almost every organ was involved in one or more patients. Infection was the most common cause of death.

Pemphigus Vulgaris. An attempt to determine whether or not a virus is the etiologic agent in pemphigus was made. Three patients with pemphigus have been studied with serologic tests, tissue culture, and electron microscopic methods. To date, all have been negative.

Other clinical studies by our branch include the first case of graft-versus-host disease in which the usual morbilliform skin eruption progressed in severity and eventuated in Toxic Epidermal Necrolysis.

Serial No. NCI-3607
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Tissue Culture Studies of Human Lymphocytes

Previous Serial Number: Same

Principal Investigator: Jay H. Robbins, M.D.

Other Investigators: W. R. Levis, M.D.

Cooperating Units: Rosemary Cuddy, Program Analysis and Reporting Section, EA, NCI. John J. Gart, Mathematical Statistics and Applied Mathematics Section, NCI.

Man Years (July 1, 1971 through June 30, 1972)

Total:	1.3
Professional:	0.6
Other.	0.7

Project Description:

Objectives

1) To investigate the mechanisms concerned with the control of mitosis, differentiation, function, and potentialities of normal and pathologic cells, with particular reference to the lymphocyte and cells of the lymphoreticular system.

2) To investigate the functional and morphological interactions between lymphocytes and macrophages and among different populations of lymphocytes during in vitro immune responses related to delayed hypersensitivity and to homograft reactions.

3) To detect, characterize, and isolate biologically active substances, including soluble mediators of cellular immunity, produced in stimulated and unstimulated human leukocyte cultures. To study their effects on supporting, augmenting, and inhibiting blastogenesis, cell division, modulation, and differentiation in vitro.

4) The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: Nature and modification of precancerous state and determination of mechanisms accounting for high degrees of stability of cell functioning.

Material:

Lymphocytes and other cells are obtained from hospitalized patients and outpatients.

Methods Employed:

Human peripheral blood lymphocytes are cultured *in vitro* and stimulated to transform into large blastoid cells by factors such as phytohemagglutinin, antigens, and foreign tissue. This phenomenon of blastogenesis is evaluated by the morphologic and biochemical changes which the lymphocytes undergo. The mechanisms by which these blastogenic factors act and the culture conditions required for blastogenesis are determined.

Human peripheral blood leukocytes are separated into a glass-adherent cell population, composed largely of macrophages, and into a population consisting of small lymphocytes. By recombining such populations obtained from the same and from different individuals, the function of each population is determined. Interactions and contacts between cells are examined by light and by transmission and scanning electron microscopic techniques, including methods involving the *in situ* fixation and embedding of the cultured cells in order to preserve the intercellular contacts and spatial relationships of the cells.

Major Findings:

Methods for obtaining 'purified' lymphocytes, glass-adherent mononuclear cells, and a population containing both cell types from human peripheral blood have been developed and analyzed. We have improved and utilized a very rapid and simple 5-minute centrifugation method using heparinized whole blood for obtaining a population of mononuclear leukocytes (lymphocytes and monocytes) with very few contaminating granulocytes. This mononuclear leukocyte population contains the two cell types required for the blastogenic response and provides cultures capable of responding with maximal responses to specific and non-specific blastogenic factors. Parameters affecting the millipore filter assay technique for measuring tritiated thymidine incorporation into DNA in leukocyte cultures have been studied and analyzed. The applications and limitations of the assay method have been determined, and it has been successfully utilized in detecting DNA synthesis in microcultures containing less than 5×10^4 leukocytes.

The performance of a specifically designed autoclave has been evaluated after its initial years of operation. This autoclave has a unique stainless steel steam generator which permits the use of distilled water to generate steam virtually free of organic or ionic contaminents which might otherwise be deposited on tissue culture glassware or utensils during sterilization. The recognized advantages of this autoclave have led to its use in several other tissue culture laboratories at the National Institutes of Health and elsewhere.

Morphological studies of cellular interaction among mononuclear cells in

leukocyte cultures have resulted in an experimental model and mathematical analysis of cell cluster formation. It is now possible to detect, measure and analyze such cluster formation not only in PHA-stimulated cultures but also in antigen-stimulated cultures as early as 2 hours after the start of incubation. Functional studies of supernates from unstimulated macrophage and leukocyte cultures have revealed the presence of a soluble factor(s) which can restore blastogenic responses to 'purified' lymphocytes stimulated either with antigens or allogeneic 'purified' lymphocytes in the absence of added macrophages. Supernates from PHA-stimulated leukocyte cultures have been studied for their ability to inhibit DNA synthesis in leukocyte cultures. These biologically active factors are to be characterized, purified and isolated.

Significance to Bio-Medical Research and the Program of the Institute:

The lymphocyte is a cell involved in immune reactions which may be operative in the immune response to neoplastic tissue. Our studies concerned with blastogenesis and the control of cell division and differentiation of the lymphocyte may elucidate this cell's normal and abnormal functions in the immune response involved in the host's defense against cancer. These studies should also provide information concerning the control of cell differentiation and division.

Proposed Course of Project:

Continuation of research as indicated in the foregoing.

Honors and Awards: None

Publications:

1. Robbins, J. H. and Jones, G. A.: An autoclave for tissue culture laboratories which sterilizes with highly purified steam. Lancet, 2: 1236-1237, 1971.

2. Robbins, J. H., Gart, J. J., Levis, W. R., and Burk, P. G. The millipore filter assay technique for measuring tritiated thymidine incorporation into DNA in leukocyte cultures. Clinical and Experimental Immunology. (In press).

3. Levis, W. R. and Robbins, J. H. Methods for obtaining 'purified' lymphocytes, glass-adherent mononuclear cells, and a population containing both cell types from human peripheral blood. Blood. (In press).

Serial No. NCI-3612
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mycosis Fungoides: Prognostic Features, Autopsy Findings, and Response to Therapy

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Joseph D. Croft, Jr., M.D., Ervin H. Epstein, Jr., M.D. and David Levis, M.D.

Cooperating Units: Biometry Branch, NCI

Man Years (July 1, 1971 through June 30, 1972)

Total:	.01
Professional:	.01
Other:	0

Project Description:

Objectives:

To review our experience with patients with mycosis fungoides.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 5.4: Improve diagnostic procedures for cancer and high and low risks, for localization of lesions.

Methods Employed:

Charts of all 144 patients with mycosis fungoides seen at the NIH have been carefully reviewed and pertinent information has been extracted. The latter has been transferred via punch cards to computer tape. With a punch card sorter and computer, appropriate correlations and summaries are being made.

Major Findings:

This group comprises by far the largest series of patients with mycosis fungoides that has been studied. Data so far accumulated indicates that survival could not be predicted by the patients' sex or race but that some other factors including age at diagnosis, duration of lesions before

diagnosis, and type of lesions at time of diagnosis all are useful prognostic features. Survival of patients after diagnosis was approximately the same during the years 1956 to 1962 as during the years 1963 to 1968. Correlating with this is the finding that long-term survival is not dramatically different whether initial treatment is with electron beam, cytoxan, or methotrexate.

Significance to Cancer Research:

By careful review of these patients, it is hoped that improved ability to predict outcome in the individual patients will result.

Proposed Course of Project

The work is completed.

Honors and Awards: None

Publications:

Epstein, E. H., Jr., Levin, D., Croft, J., and Lutzner, M. A.: Mycosis Fungoides: Survival, Prognostic Features, Response to Therapy, and Autopsy Findings. Medicine. 51: 61-72, 1972

Serial No. NCI-3613
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Extraction, Isolation and Characterization of Keratohyalin Granules.

Previous Serial Number: Same

Principal Investigator: Arthur Ugel, M.D.

Other Investigators: Drs. Rodbard and Chrambach.

Cooperating Units: Endocrinology Branch, NCI.

Man Years (July 1, 1971 through June 30, 1972)

Total:	3.4
Professional:	1.4
Other:	2.0

Project Description:

Objectives:

1) To verify the isolation of keratohyalin (KH) by immunological methods.

2) To characterize the physical-chemical properties of bovine KH.

3) To compare the histochemical, ultrastructural and physical-chemical properties of bovine KH when isolated by various salt solutions.

4) To further demonstrate homology of bovine KH by reversibility studies and amino acid analyses of the various bovine KH oligomeric states.

5) To demonstrate homology of bovine KH oligomers by immunological methods.

6) To develop an *in vitro* system for synthesizing labeled bovine KH in order to further study the cellular site of synthesis of KH, and to determine rates of synthesis of bovine KH: To study the incorporation of tritiated amino acids into *in situ* KH granules in order to better characterize the rate of synthesis and the composition of the *in situ* KH granule.

7) To obtain an insight into the chemical structure and composition of trichohyalin through Histochemical stains and determine if an antigenic

relationship exists between trichohyalin and keratohyalin.

8) To determine if the basic protein isolated by Ugel from cow hoof originated from and was identical to keratohyalin.

9) To isolate and compare keratohyalin from other ungulates.

10) The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: Nature and modification of precancerous state and determination of mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

1) Crude bovine KH is isolated from cattle hoof epidermis by extraction with 1.0 M potassium phosphate buffer (pH 7.0). This extract is dialyzed against 32 volumes of distilled water which results in the in vitro aggregation of bovine KH. The resultant macroaggregates are injected into rabbits with Freud's adjuvant, and localization of antibody is performed by the indirect method on sections of cattle hoof epidermis and isolated macroaggregates of bovine KH.

2) Crude bovine KH is isolated by in vitro agregation as described above. The macroaggregates are solubilized in sodium decylsulfate, then subjected to polyacrylamide gel electrophoresis at various gel concentrations.

3) Crude bovine KH is isolated as described above, except that the salts used for extraction are varied. In all cases, the initial extraction salt concentration is maintained at 1.0 M. The various aggregates are studied by histochemical, ultrastructural, and physical-chemical methods as described.

4) Sodium decylsulfate solubilized bovine KH is fractionated on 9% polyacrylamide gels and the various species are rapidly precipitated by immersion in 7.5% acetic acid. The species are then sliced from gels and extracted with sodium decylsulfate. These isolated species are then subjected to polyacrylamide gel electrophoresis as described above. Other isolated species (confirmed by polyacrylamide gel electrophoresis) are precipitated with 5% trichloracetic acid, washed, and analyzed for amino acids.

5) The various species of bovine KH are fractionated and isolated as described in 4. These species are then injected into rabbits, and indirect immunofluorescence is performed as described in 1.

6) Cattle hoof epidermis is minced, then placed in Media 199, or in similar media depleted of certain amino acids. Tritiated amino acids are then added, and incorporation is continued for various time periods. Samples of epidermis are then prepared for radioautographical studies, and other samples of epidermis are extracted with 1.0 M potassium phosphate buffer (pH 7.0) as described above (1).

7) Standard histochemical stains are employed in order to determine the staining properties of trichohyalin granules. Methanol fixed, paraffin embedded sections were then prepared of hair containing epidermis in order to determine if anti-keratohyalin antibodies cross-react with trichohyalin granules.

8) The oligoribonucleoprotein isolated by Ugel were used to immunize rabbits and goats. Both crude material and highly purified material isolated from polyacryalamide gels were used. Immunofluorescent techniques were employed to determine the antigenic site of the immunizing material.

9) Keratohyalin was isolated from cow, goat, wildebeest, and sheep. Histochemical studies were employed on the isolated material, and each species keratohyalin were characterized on polyacrylamide gels. Rabbits and goats were immunized with the isolated keratohyalin, and immunofluorescent techniques were employed for antibody detection.

Major Findings:

1) Immunization of rabbits with bovine macroaggregates results in the production of antibody directed against the in situ KH granule, as well as the isolated macroaggregate. The titer can be completely abolished by absorption with the isolated macroaggregate, and by no other material investigated to date. Fluorescence is limited to the in situ KH granule, and no other areas are positive. Cross-reactivity of bovine and rabbit KH can be demonstrated by indirect immunofluorescence.

2) Polyacryamide gel electrophoresis of bovine KH results in the fractionation of a 13 member homologous series of oligomers. Inspection of the fractionated species indicates that each size oligomer migrates with a small amount of conformational isomer. There is a linear relationship between the retardation coeffient (the slope of log mobility versus gel concentration), and oligomer number. This relationship has been used to determine the size of all the species of KH. These studies indicate that the monomer (subunit) has a molecular weight of 16,900. The extrapolated free mobility of all the species is constant. Crude bovine KH when fractionated on 9% polyacrylamide gels, also shows the presence of two nonhomologous species which appear to be conformational isomers of the smaller oligomers. By varying the gel concentration and selectively unstacking these two nonhomologous species in 3% polyacrylamide gels, it has been possible to purify and isolate the entire group of 13 species in one step. The theoretical model developed to fit these data indicates that bovine KH should exist as a linear array of random coils in the presence of anionic detergent.

3) Regardless of the salt used to extract bovine KH, in vitro aggregation results in the formation of macroaggregates with histochemical characteristics of the in situ KH granule. At the ultrastructural level, those aggregates formed from 1.0 M potassium phosphate extracts are the most ordered and homogenous. Polyacrylamide gel electrophoresis of the various aggregates (solubilized in sodium decylsulfate) indicates that

the KH obtained from potassium phosphate buffer is composed only of the homologous series of oligomers.

4) Isolation of high molecular weight oligomers of bovine KH, and refractionation results in the demonstration of the original high molecular weight oligomers, but small amounts of the lower molecular weight species are also present, and moderate amounts of the bovine KH monomer are seen. At no time can polymerization into higher molecular species be demonstrated. Amino acid analyses (corrected for loses of serine and tyrosine) and for tryptophan are identical for all the various species of bovine KH. The molecular weight of the subunit (monomer) of KH from these studies is 14,300. The analyses indicates a unique composition in the large amounts of serine, histidine, arginine and glycine that are present.

5) Isolation of the various species of bovine KH (as described above) and immunization of rabbits with these species results in the production of antibody to the entire in situ KH granule and the isolated macroaggregate.

6) Incorporation of tritiated histidine into bovine KH demonstrates label as early as 5 minutes, as demonstrated by isolation of labeled KH and autoradiographical methods. Incorporation continues at a linear rate for at least 24 hours. At no time is there labeling of the nucleus.

7) The antibody to keratohyalin reacts with trichohyalin, indicating that chemical similarities exist between the two substances.

8) Antibodies could be made to in situ keratohyalin granules indicating that the isolated material is keratohyalin. Antibodies cross-react with rabbit, sheep, wildebeest, and goat keratohyalin granules but not with rat, guinea pig, or human keratohyalin. Confirmation that the protein is an oligomeric series was obtained.

9) All species had a protein isolated with identical histochemical characteristics. All species had identical polyacrylamide gel characteristics demonstrating a polymeric series of 20 or more polymers. The protein isolated from each species produced antibodies in rabbits which were directed against in situ keratohyalin granules, indicating that the isolated material was keratohyalin. However, bovine keratohyalin immunized rabbits formed antibodies cross-reacting with sheep, goat, wildebeest, and rabbit. But bovine keratohyalin immunized goats formed antibodies directed against bovine keratohyalin only. Blocking experiments with rabbit antibovine KH and goat antibovine KH confirmed these findings. Thus, although histochemical and physical chemical studies show the four species to have the same kind of KH immunological studies indicate that some antigenic sites are different, perhaps indicating that the molecules have different configurational structures.

Significance to Cancer Research:

Presently, the function of KH is completely unknown. However, there are certain reasons for believing that KH may have a role as a "control" substance, based on both clinical and biochemical observations. Clinically, diseases of the skin which are characterized by atypical mitotic rates and fast turnover times (basal cell and epidermal cell cancer and psoriasis) are remarkable for the absence of morphologically recognizable KH they demonstrate at the histopathological level. A hypergranular skin, on the other hand, is usually one with a slow turnover rate. In resolving psoriasis, return of KH is noted. Biochemically, KH is composed of a basic protein complexed to RNA. It is entirely possible that the bound RNA plays a role in controlling the epidermal cell, and that the basic protein acts to specifically bind acidic nucleotides, possible turning off certain genes.

Proposed Course of Project:

Research will be continued along these lines.

Honors and Awards:

Gold Award for scientific exhibit: "Isolation and characterization of bovine keratohyalin", American Academy of Dermatology, Chicago, Illinois, Dec., 1971.

Publications:

1) Ugel, A.R.: Isolation and characterization of oligoribonucleoproteins with histochemical and ultrastructural characteristics of keratohyalin. J. Cell Biol. 49: 405-422, 1971.

2) Ugel, A.R., Chrambach, A., and Rodbard, D.: Characterization of an Oligomeric Series of bovine keratohyalin by polyacrylamide gel electrophoresis. Anal. Bioch. 43: 410-426, 1971.

3) Ugel, A.R. and Idler, W.W.: Further Characterization of Bovine Keratohyalin. J. Cell Biol. 52: 453-464, 1972.

4) Elias, P.M., Montague, P.M. and Ugel, A.E.: In Vitro Studies on the Kinetics, Composition, and Homology of Bovine Keratohyalin. (In press).

5) Guss, S.B. and Ugel, A.R.: Immunofluorescent Antibodies to Bovine Keratohyalin and Immunologic Confirmation of Homology. J. Histochem. Cytochem. 20: 97, 1972.

Serial No. NCI-3617
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Electron Microscopic Study of the Ultrastructure of Darier's Disease.

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Sheldon K. Gottlieb, M.D.

Cooperating Units: Massachusetts General Hospital, Boston, Mass.

Man Years (July 1, 1971 through June 30, 1972)

Total:	.01
Professional:	.01
Other:	0

Project Description:

Objectives:

To study the ultrastructure of skin in the autosomal dominant genodermatosis, Darier's Disease, in an attempt to determine the derivation of the corps ronds and the grain with the intention of obtaining clues as to the possible chemical defect in this puzzling disease.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 4.1: Biology of cells capable of forming cancers - steps preceding cancer development at cell transformation (and other) body sites: determine rates of (cancer) cell transformation and percent of rakes leading to tumor formation.

Methods Employed:

Patients are sought with Darier's Disease, punch biopsies are taken and tissue is processed for electron microscopy.

Major Findings:

19 lesions from 5 patients of 3 families with Darier's Disease were biopsied and studied by light and electron microscopy. Large cytoplasmic vacuoles were seen in basal cells. These vacuoles accumulated in in-

creasing numbers in prickle and granular cells, which became acantholytic. In the granular layer, some cells were expanded by vacuoles which compressed existing tonofilaments and other cytoplasmic into a peripheral ring. These heavily vacuolated cells correspond to corps ronds. Other vacuolated cells continue to keratinize as evidence by formation of keratohyalin granules and aggregated bundles of tonofibrils which finally compressed the vacuoles into slits. These cells become ovoid, retained their nuclei and correspond to grains. Thus the present study indicates that grains and corps ronds can both originate from vacuolated keratinocytes. The gene defect in Darier's Disease may be closely related to the formation of this abnormal cytoplasmic vacuole.

Significance to Cancer Research:

Further study of genetic mechanisms in human genodermatosis should enable one to determine how normal keratinocytes operate and thus may eventually give clues as to the development of squamous cell and basal cell carcinoma.

Proposed Course of Project:

Terminated

Honors and Awards: None

Publications:

Gottlieb, S. K. and Lutzner, M. A.: Darier's Disease: An Electron Microscopic Study. Arch. Derm. (In press).

Serial No. NCI-3623
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Evaluation of Various Elastic and Collagen Tissue Diseases

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Thomas Nigra, M.D., Michael Friedland, M.D., Michael Sussman, M.D. and Jack Lichtenstein, M.D.

Cooperating Units: National Institute of Dental Research, Connective Tissue Section, Biochemistry Branch.

Man Years (July 1, 1971 through June 30, 1972)

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

Objectives:

Purpose of the project is to determine steps involved in the formation of normal connective tissue as a baseline for study of disease states. Particular emphasis is placed on the chemistry of collagen and elastin, the enzymatic steps of cross linking, and the degradative processes.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: Nature and modification of precancerous state and determination of mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

The usual biochemical and tissue culture techniques in studying collagen metabolism are employed.

Major Findings:

1) Collagenase is the major degradative enzyme found to date for the collagen molecule. This enzyme has been found in a number of tissues. Heretofore, no one has identified this particular neutral degradative

enzyme in human diploid fibroblast in culture. We have isolated this enzyme in the culture system and are currently attempting further characterizing its nature.

2) Collagen biosynthesis fibroblast culture - In fibroblast culture the various factors affecting the rate of collagen synthesis are being examined. Particularly at this time the effect of pH on precursors of collagen biosynthesis and on the enzymes of collagen biosynthesis is being examined in collaboration with Dr. Harry Eagle of Albert Einstein Medical College. Recent data indicate that there is a 2-fold increase in protein synthesis and a 4-fold increase in collagen synthesis at pH 7.8 as compared to pH 7.4.

3) Pinnel, et al have described an inherited hydroxylysine deficient collagen disease of human skin in one kindred. We have recently found a second family with this hereditary disorder of connective tissue. Both families had the typical clinical appearance of Ehlers-Danlos Syndrome. We are currently examining the biochemical ramifications of this collagen disease in order to determine if the two families have the same biochemical defect.

4) The effects of acute and chronic inflammation on collagen synthesis and degradation are being studied. Peptidyl proline hydroxylase and collagenase activity has been measured sequentially in the Arthus reaction, the passive Arthus reaction and the delayed hypersensitivity reaction. Peaks of enzyme activity are reproducible from animal to animal but vary with the nature of the lesion (acute or chronic inflammation). In the Arthus reaction there is a peak of collagenase activity 12 hours after initiating the lesion. Immune complexes are present; polymorphonuclear cells are maximal at this time. Furthermore, it has been shown that cells, in vitro, release collagenase in the presence of complement by immune complexes.

Significance to Cancer Research:

Collagenase production by malignant cells may be related to tumor invasiveness and metastasis.

Proposed Course of Project:

Ongoing

Honors and Awards: None

Publications:

Nigra, T., M.D., Friedland M., M.D., and Martin G., M.D.: Control of Connective Tissue Synthesis: Collagen Metabolism. J. Invest. Derm. (In press).

Serial No. NCI-3629
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Use of Osmium Zinc Iodide (O_sO_4 - ZnI_2) as a Special Stain for the Golgi Apparatus and Other Subcellular Organelles.

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Peter M. Elias, M.D., Bruce K. Wetzel, Ph.D.

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	.7
Professional:	.2
Other:	.5

Project Description:

Objectives:

To determine the pattern of organelle staining in hydras by variations of the O_sO_4 - ZnI_2 method.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 4.2: Biology of cells capable for forming cancer and interactions which host cells - precancerous metabolic and morphologic lesions.

Methods Employed:

All material is eventually studied by electron microscopy. Initially, the tissue is exposed for varying time periods to different concentrations and substituted combinations of O_sO_4 - ZnI_2, phosphate buffer, and other cationic metallic-substances; then, it is routinely processed for EM.

Major Findings:

1. Following 4 hours, combined fixation-staining with O_sO_4 - ZnI_2, the stain was limited to the Golgi apparatus and certain surface coats of Hydra littoralis and Hydra pseudoligactis. Adjustment of the

staining solution to neutral pH with .1NNaOH results in staining limited to the Golgi apparatus. Shorter or longer staining with OZI produces only partial Golgi staining and absence of surface coat staining.

The luminal surfaces of large intracellular vacuoles were also delineated under certain conditions, and staining in some sites is abolished by pretreatment with sialidase or hyaluronidase, suggesting the involvement of acid mucosaccharides in sites which stain with OZI. Elongated coated vesicles of Hydra epithelium reportedly analogous with the enigmatic Odland bodies of human epidermal cells, have failed to stain with OZI, thus establishing a difference between these organelles.

Significance to Cancer Research:

OZI staining may be of general use in cytological studies, and the observations on Hydra epithelium are relevant to our basic understanding of skin biology.

Proposed Course of Project:

To be continued.

Honors and Awards: None

Publications:

Elias, P. M., Park, H., Patterson, A. E., Lutzner, M. A., and Wetzel, B. K.: Osmium tetroxide zinc iodide staining of golgi elements and surface coats of hydras. J. Ulstruct. Res. (In press).

Serial No. NCI-3630
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Vitamin A Acid Induced Mucous Metaplasia of Embryonic Chick Metatarsal Skin in Organ Culture: A Histochemical and Electron Microscopic Study.

Previous Serial Number: Same

Principal Investigator: Gary L. Peck, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	1.4
Professional:	0.4
Other:	1.0

Project Description:

Objectives:

1) To investigate the mechanisms governing epidermal cell differentiation.

2) To determine if epidermal cells are capable of both mucin and keratin production, or whether the two processes are mutally exclusive.

3) To study the early changes of Vitamin A acid induced mucous metaplasia at an ultrastructural level.

4) The Objective and Approach of the National Cancer Plan that this project most closely supports is 4.1: Biology of cells capable of forming cancers - steps preceding cancer development at cell transformation (and other) body sites: determine rates of (cancer) cell transformation and percent of rakes leading to tumor formation.

Material:

Metatarsal shank skin (devoid of feathers) is obtained from 13-14 day chick embryo.

Methods Employed:

Chick metatarsal skin is cultured in vitro and stimulated to undergo mucous metaplasia by Vitamin A acid at varying doses. Specimens are taken at varying time intervals and evaluated for mucin production by the use of special stains, such as PAS (with and without diastase), Alcian Blue, Toluidine Blue, and Colloidal iron. Specimens are also fixed in glutaraldehyde for subsequent examinations under the electron microscope.

Major Findings:

1) Using 30 international units of Vitamin A acid per milliliter of culture media, it was found that mucous metaplasia occurred with 2 days in in vitro incubation. Maximum metaplasia took place by 6 days.

   As little as 1 I.U. produced metaplasia in 6 days.

   We have studied specimens of chick skin harvested prior to 2 days of incubation and treated with low dosage Vitamin A in order to determine the earliest mechanism involved in mucus metaplasia. Ultrastructurally, we found no specific mechanism operative, other than increased amounts of rough endoplasmic reticulum and polyribosomes indicating increased protein synthetic activity.

2) Microscopically, the control specimens keratinized normally with the formation of thick bundles of tonofilaments, keratohyalin granules, and a multi-layered stratum corneum. Vitamin A acid treated specimens did not undergo keratinization. There was no tendency of the columnar basal cells to flatten as they approached the surface. Neither stratum corneum, nor keratohyalin granules were formed. Tonofilaments were present in small, fine bundles. In addition, there was increased folding of the nuclear membrane, and increased amounts of rough endoplasmic reticulum and polyribosomes. Mucin was present in the cytoplasm as amorphous droplets of electron dense material. These electron dense bodies appear similar to lysosomes; however, these structures are acid-phosphatase negative.

3) In addition to mucin formation there seems to be a glandular metaplasia in the epidermis with formation of acini. This morphologic change has not been demonstrated previously. Our standard system now utilizes 13-14 day old chick embryos treated with 20 i.u. Vitamin A acid in Eagle's Minimal Essential Media with 5% fetal calf serum. Cultures are harvested at 2-3 days.

4) Vitamin A induced mucus glandular metaplasia was inhibited by the addition of puromycin or cycloheximide to the culture medium - actinomycin D, hydroxyurea, and colchicine failed to inhibit this process. Thus, protein synthesis is necessary for metaplasia, while mitosis, and DNA and RNA synthesis are not.

Significance to Cancer Research:

Knowledge of cell differentiation is basic to the understanding of malignant processes.

Proposed Course of Project:

1) Continued ultrastructural examination of the above material, including scanning electron microscopy.

2) The effect of Vitamin A acid on epidermal cell kinetics, mucin biosynthesis, and protein metabolism will be made by observing the incorporation of tritiated precursors by chick metatarsal skin.

3) Chemical and immunological characterization of epidermal mucin.

Honors and Awards: None

Publications: None

Serial No. NCI-3631
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Treatment of Skin Cancer with Topical BCNU, and CCNU.

Previous Serial Number: Same

Principal Investigator: Gary L. Peck, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total: .1
Professional: .1
Other: 0

Project Description:

Objectives:

To evaluate the therapeutic effectiveness and toxicity of BCNU and CCNU administered topically to the skin surface of the above patients.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 6.1: Develop and utilize methodologies of immunology chemotherapy, surgery and radiation (alone) and in combinations in the cure and palliation of cancer.

Agents:

0.1%, 0.2% and 0.4% Bis-Chloro-ethyl-nitrosourea; and 0.1%, 0.4% and 1.0% chloroethyl-nitrosourea; in 30% liquid petrolatum, 70% white petrolatum.

Material:

1) Basal Cell Carcinoma

2) Nevoid Basal Cell Carcinoma Syndrome

3) Xeroderma pigmentosum with cutaneous carcinoma.

Methods Employed:

1) Patients must have pathologic confirmation of the clinical diagnosis.

2) They must have normal hematologic, hepatic, and renal function.

3) Lesions on eyelids and canthi are not included.

4) Patients are cautioned to avoid sun.

5) Ointments are applied twice daily to affected areas for 8 weeks.

6) Laboratory tests are done before, during, and 6 weeks after therapy.

7) Serial photographs are taken.

Major Findings:

1) A total of 12 patients were included in this study. Nine received only BCNU ointment. One received only CCNU ointment; the remaining two received CCNU ointment initially and were given BCNU later as a second course of therapy. Five of the 12 patients were treatment failures by other modalities, including surgery and topical 5-FU in propylene glycol. Four of the patients had had X-ray therapy of their acne earlier in life. Four other patients had moderate to severe actinic skin. Two patients had the basal cell nevus syndrome: one had xeroderma pigmentosum. Most patients had lesions of the exposed areas of the face and neck. Many patients also had lesions on the trunk and extremities. None of the 12 had the standard variety of BCE histologically; the remaining three were superficial BCE, morphea type of BCE, and the fibroepithelioma type of BCE (Pinkus). There were nine males, three females, varying in age from 23-65.

2) Results of treatment are as follows:
BCNU 0.1% 26 lesions 10 cures 15 partial responses one no response
CCNU 1.0% 7 lesions 1 cure 5 partial responses one no response
Partial responses is defined as visible flattening of the tumor mass.
The ten BCNU cures were in only four patients.

3) To date, neither recurrences, nor signs of chronic toxicity have been observed.

4) Severe contact dermatitis has occurred in some patients with 0.1% BNCU ointment. The dermatitis appeared from day 2 to day 33 in the different patients. Several patients have not developed any cutaneous irritation. In three patients tumors disappeared without antecedent skin inflammation. Usually the patients who had no skin reaction to the medication had little to no therapeutic response. This supports the clinical impression derived from experience with topical 5-FU: No inflammation, No Response.

5) The main difficulty in conducting this study was the lack of an assay system for the stability of the ointment. The active drugs are unstable in water or propylene glycol, therefore, petrolatum is the only suitable

vehicle, and it is not possible to extract the active drug from the petrolatum ointment. This made establishing of the shelf-life difficult. By experience we determined that ointments over one month were not stable.

Significance to Cancer Research:

1) This is an exploratory study of a new drug in skin cancer prompted by a desire to find a superior agent to 5-Fluorouracil in propylene glycol which is in widespread topical use.

2) We would also hope to contribute to the knowledge of the clinical pharmacology of this agent. Skin cancer should prove to be a valuable lesion for the screening of this and future topical chemotherapeutic agents becaus the tumors are observable, measurable, and, therefore, can be used as an objective indicator of response to therapy. Furthermore, the patients can be expected to be in good health, which would allow for long term studies.

Proposed Course of Project:

1) Because of the variability in response to the drug and the lack of a stability assay for the ointment, this study has been discontinued.

2) Discontinuance of CCNU ointment as it produced only partial responses in spite of prolonged therapeutic courses.

3) Continued observation of test subjects to determine recurrence rate and for any signs of chronic toxicity.

Honors and Awards: None

Publications: None

Serial No. NCI-3632
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Toxic Epidermal Necrolysis and Graft-Versus-Host Reaction

Previous Serial Number: Same

Principal Investigator: Gary L. Peck, M.D.

Other Investigators: P. M. Elias, M.D., G. P. Herzig, M.D.

Cooperating Units: Medicine Branch, NCI

Man Years (July 1, 1971 through June 30, 1972)

Total:	.05
Professional:	.05
Other:	0

Project Description:

Objectives:

To investigate the relationship between Graft-Versus-Host Reaction (GVH) and Toxic Epidermal Necrolysis (TEN).

The Objective and Approach of the National Cancer Plan that this project most closely supports is 6.1: Develop and utilize methodologies of immunology, chemotherapy surgery and radiation (alone) and in combinations in the cure and palliation of cancer.

Methods Employed:

1) Taking of history, and repeated physical examinations of a patient with acute lymphocytic leukemia, treated with allogeneic bone marrow transplantation, who developed GVH, and subsequently, TEN.

2) Review of literature

3) Examination with the electron microscope of multiple skin biopsies taken at various time intervals and at various skin sites.

Major Findings:

1) This is the first documented case in which the usual morbilliform erup-

tion associated with sheet-like desquamation of the epidermis, typical of TEN.

2) Early in the clinical course, cutaneous histopathology was typical for GVH.

3) Later in the clinical course, biopsies taken from intensely erythematous sites, near areas of massive epidermal desquamation showed more extensive epidermal destruction, consistent with the diagnosis of TEN.

4) Support for our thesis linking TEN in this patient with his GVH, comes from animal studies in which experimental GVH in hamsters and monkeys developed TEN. Furthermore, a case of GVH was found in the literature in which a bullous eruption, in retrospect compatible with TEN, developed.

5) Electron microscopic findings: In biopsies taken at distances greater than 10 cm. form the blister edge, ultrastructural changes were limited to the basal cell layer. The entire basal layer was involved in a uniform manner, while the spinous, granular, and horny layers appear virtually uninvolved. Mitochondrial swelling coarsening of tonofibrils, and increased prominence of ribosomes was noted in all basal cells. The nuclei appeared contracted, with more convoluted nuclear membranes; increased density of chromatin was noted. Biopsies closer to the blister edge displayed considerable intercellular edema, and occasional early bulla formation. Lymphocytes were occasionally seen within the basal layer, but only rarely above. Increased numbers of Odland bodies and abnormal keratohyalin granules were noted in the superficial epidermis. In summary, the initial pathologic events are in the basal layer, and it appears that the nomonuclear infiltrate may play a significant pathogenetic role.

Significance to Cancer Research:

Immunosuppressed patients, including treated cancer patients, who are transfused or transplanted with immunocompetent cells, may expect to develop GVH. In view of this case report, we may now expect some of them to develop TEN as well.

Proposed Course of Project:

Electron microscopic examination is continuing. Specimens from patients with either TEN, or GVH are also being studied.

Honors and Awards:

First Prize - 1971 North American Clinical Dermatological Society Memorial Essay Contest.

Publications:

Peck, G. L., Herzig, G. P., and Elias, P. M.: TEN in a patient with GVH Arch. Derm. 105: 561-569, 1972.

Serial No. NCI-3633
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Treatment of Psoriasis with Topical CCNU. (IND-7003)

Previous Serial Number: Same

Principal Investigator: Gary L. Peck, M.D.

Other Investigators: Stephen Guss, M.D. and Douglas Key, M.D.

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	.15
Professional:	.15
Other:	0

Project Description:

Objectives:

To evaluate safety and effectiveness of topical CCNU in psoriatic patients.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 6.1: Develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (alone) and in combinations in the cure and palliation of cancer.

Agents:

0.4% and 1.0% CCNU in 30% liquid petrolatum and 70% white petrolatum.

Material:

Adult patients with psoriasis. Females must be past the childbearing age.

Methods Employed:

1) Patients must have psoriasis previously unresponsive to standard therapy.

2) They must have normal kidney, liver, and bone marrow function.

3) Medication will be applied twice daily until clear.

4) Patients will avoid sun exposure to prevent phototoxicity, if any.

5) Laboratory tests measuring bone marrow, liver, and kidney funtion will be obtained before, during, and 6 weeks after cessation of therapy.

6) Serial photographs will be obtained.

Major Findings:

1) Seven of 9 patients treated for longer than 2 weeks cleared completely. The 2 patients who did not clear had severe psoriasis of the palms and soles. Four additional patients were treated for less than 2 weeks.

2) Severe irritation of the skin surrounding the psoriatic plaques occurred. This was especially marked in the intertrigenous areas.

3) Subsequent to the cutaneous irritation, persistent pigmentation, telangiectasia, and cutaneous pain developed as well as temporary bone marrow suppression.

4) Four patients have remained free of disease since October, 1970 to the present.

5) No signs of delayed or chronic toxicity have appeared.

Significance to Cancer Research:

1) This study is part of an ongoing search for an effective topical therapy for psoriasis.

2) It is hoped that this study will contribute to the pharmacology of chemotherapeutic agents.

Proposed Course of Project:

1) Because of the local and systemic toxicity, this study has been discontinued.

2) Continued observation of test subjects - for any chronic toxicity and for determination of length of remission.

Honors and Awards: None

Publications:

Peck, G. L., Guss, S. B. and Key. D. J. Topical CCNU in the Treatment of Psoriasis. Arch. Derm. (In press).

Serial No. NCI-3635
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Investigation of lysosomal activity in Epidermolysis Bullosa Simplex (EBS)

Previous Serial Number: Same

Principal Investigator: Gary L. Peck, M.D.

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	.05
Professional:	.05
Other:	0

Project Description:

Objectives:

To investigate the pathogenesis of blister formation in EBS.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 4.1: Biology of cells capable of forming cancers - steps preceding cancer development at cell transformation (and other) body sites: determine rates of (cancer) cell transformation and percent of rakes leading to tumor formation.

Material:

1) Skin from patients with EBS

2) Skin from patients with EB dystrophica, xeroderma pigmentosum, and normals, to serve as controls.

3) Mouse liver.

Methods Employed:

1) Biopsies from traumatized and non-traumatized uninvolved skin of patients with EBS are fixed in 6% GTA in phosphate buffer for routine ultrastructural examination.

2) Similar specimens fixed in 6% GTA in cacodylate buffer will be examined for acid phosphatase activity.

3) Control tissues will be processed similarly.

Major Findings:

1) Uninvolved, non-traumatized skin of EBS is abnormal in 2 respects:

   a) there is occasional basal cell degeneration with marked intercellular edema, fragmentation of tonofilaments, and dissolution of other intracytoplasmic organelles.

   b) there is increased melanin pigment transfer from melanocytes to keratinocytes with formation of giant membrane-bound melanosome complexes.

2) These melanosome complexes are acid phosphatase positive. This has been reported in the literature and these complexes have been called lysosomes. No other acid phosphatase positive organelles are seen in our specimens.

3) However, appropriate acid phosphatase controls are also positive (incubation with sodium fluoride, omitting the substrate B-glycerophosphate from the incubation mixture, and even incubation of the tissues in lead nitrate alone).

4) Acid phosphatase reactions in xeroderma pigmentosum, EB dystrophica, norma skin and mouse liver react similarly.

5) Under routine ultrastructural examination, xeroderma pigmentosum differs from EBS by not forming large melanosome complexes; EB dystrophica produce intradermal blisters, and, as such, there is no basal cell degeneration.

6) Topical Vitamin A acid had no effect on the E.M. findings or on the clinical disease, although Vitamin A is known to labilize lysosomal membranes.

Significance to Cancer Research:

1) Basic understanding of melanin physiology.

2) Elucidation of the significance of the acid phosphatase reaction in human skin.

3) Confirmation of published work on electron microscopy of EBS.

Proposed Course of Project:

Continued EM observations

Honors and Awards: None

Publications: None

Serial No. NCI-3636
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Susceptibility of fibroblasts from xeroderma pigmentosum patients to oncogenic virus transformation post-irradiation in vitro.

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Douglas Key, M.D. and Stuart Aaronson, M.D.

Cooperating Units: Viral Carcinogenesis Branch

Man Years (July 1, 1971 through June 30, 1972)

Total:	.97
Professional:	.97
Other:	0

Project Description:

Objectives:

Xeroderma pigmentosum is a genodermatosis with a marked predisposition to development of carcinomas in sun exposed areas. A defect in DNA repair, post UV-damage, has been shown in most of these patients. The question arises as to whether the carcinogenesis shown in these patients is perhaps the expression of oncogenic virus activity initiated or allowed by the suspected enzyme defect in UV-DNA repair in these patients. The purpose of this study is to obtain parameters of both DNA and RNA oncogenic virus infection and transformation of fibroblasts from normals and patients with xeroderma pigmentosum after varying dosages of ultra-violet radiation.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.2: Nature and rate of oncogenic cell transformations in carcinogenesis.

Methods Employed:

Fibroblasts as grown out from skin biopsy explants to undergo SV-40, a DNA virus, transformation assay as described. Infectivity to be determined by immunofluorescent T antigen incidence; transformation by gross colony counts. Cell survival needed for obtaining the fraction of total colonies

which are transformed will be determined by direct colony counts on feeder layers. Additional attempts will be made to perform parallel studies with RNA oncogenic focus forming viruses.

Major Findings:

With SV-40 virus infection post-irradiation of both normal and xeroderma fibroblasts there was no enhancement of transformation, but a decrease in transformation in proportion to the post-irradiation survival curve. The T antigen incidence, as a rough marker of infectivity was the same in irradiated as non-irradiated cells as measured 48 hours post infection.

Significance to Cancer Research:

The possible demonstration of enhanced susceptibility after UV irradiation to transformation by oncogenic viruses in human cells would be an exceedingly valuable in vitro model in view of: 1) the clinical predisposition to carcinogenesis in sun exposed areas in this rare genodermatosis; 2) Possible relationship to predisposition of older populations to sun-induced cutaneous malignancies.

Proposed Course of Project:

Initial attempts to establish a RNA oncogenic focus forming virus infection in xeroderma cells proved unsuccessful. However, studies are now underway with the newly suspected primate agents. If xeroderma cells should prove susceptible to such an infection then parallel studies will be done as described.

Honors and Awards: None

Publications: None

Serial No. NCI-3637
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Melanogenesis in Normal and Abnormal Mammalian Tissues

Previous Serial Number: Part of this work has been under NCI-3617

Principal Investigator: Vincent J. Hearing, Jr., Ph.D.

Other Investigators: Marvin A. Lutzner, M.D.

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	1.0
Professional:	1.0
Other:	0.0

Project Description:

Objectives:

1) To examine melanogenesis in various pigment forming tissues both in normal and abnormal conditions by electron microscopy.

2) To characterize the structural composition of melanin granules throughout their development in vivo.

3) To investigate the control mechanisms involved in regulating melanogenesis in mammals.

4) The Objective and Approach of the National Cancer Plan that this project most closely supports is 4.2: Biology of cells capable for forming cancer and interactions which host cells - precancerous metabolic and morphologic lesions.

Methods Employed:

1) Tissues selected for examination were dissected out and fixed in glutaraldehyde, then osmium, dehydrated, embedded, sectioned and stained by conventional procedures. Specimens were then critically examined both by light and electron microscopy.

2) To date our research has been aimed primarily at elucidation of the

structure of mammalian melanin granules through examination of those of the mouse. Eyes from inbred mouse mutants were excised, minced in 0.25 M sucrose and the melanin granules subsequently isolated by an extensive series of differential centrifugations. Granules thus isolated are solubilized in one of several solvents, then either subjected to polyacrylamide gel electrophoresis, ion exchange chromatography and/or density gradient ultracentrifugation.

3) Tyrosinase activity has been investigated both by spectrophotometric and radioactive assay methodology. A tissue homogenate to be examined is incubated with tyrosine and DOPA (dihydroxyphenylalanine) (either radioactive or cold) along with assorted translational inhibitors. The production of melanin from tyrosine and DOPA by action of the enzyme tyrosinase can be followed spectrophotometrically at 475 nm or, after repeated washings, by liquid scintillation counting of newly formed radioactive melanin.

Major Findings:

1) A series of pigment mutants of the mouse has been investigated thus far which are analogues of normal and abnormal human pigmentation: black (normal), white (albinism) and beige (Chediak-Higashi syndrome), among others. The results of this phase of our work indicate that a common pathway of melanogenesis exists, which is common to all pigment mutants studied. A fibrillar premelanosome is formed, upon which melanin is uniformly deposited. Eventually the melanized fibers become so thick that fusion between them apparently takes place, and all structural detail is masked. In the mutants studied, the basic difference from normal melanogenesis as described above is the premature termination of melanin synthesis at one of several points. This results in incompletel formed, rather than aberrant, granules. One exception to this is found in the beige animal, in which many normally forming granules fuse together, resulting in giant granules. Since the fusion of other types of granules in these beige animals in noted, the mechanism of fusion is not restricted to pigment granules alone.

2) Preliminary results indicate that the normal melanin granule is composed of only five proteins, four of which are loosely bound (most likely forming the limiting membrane), the other being tightly bound within the melanin polymer matrix (presumably the fibrillar structural protein).

3) Since only one enzyme (tyrosinase) is essential for melanin biosynthesis it is a unique system for the study of enzymatic control mechanisms. We have examined tyrosinase activity in normal (black) animals and albino animals, which have been reported to have no activity (which results in a complete lack of melanin synthesis). We have found, interestingly enough, that albinos do produce some tyrosinase, but it is somehow block from its activity *in vivo*. One can demonstrate this activity *in vitro* in albinos by treatment of the tissue with detergent (Triton X-100) or with RNase, but not, significantly, with any proteases. On the contrary when one treats normal tissue with these reagents, one finds different characteristics. RNase elicits a slight increase in activity, while

in albinos by treatment of the tissue with detergent (Triton X-100) or with RNase, but not, significantly, with any proteases. On the contrary, when one treats normal tissue with these reagents, one finds different characteristics. RNase elicits a slight increase in activity, while Triton X-100 inhibits tyrosinase activity in normal animals by approximately 80%. Proteases, on the other hand, result in a large increase in activity. These results indicate inhibition control systems for tyrosinase activity in black and albino animals differ greatly, and in addition, that much work is necessary to properly evaluate them.

Significance to Cancer Research:

The characterization of normal mammalian melanogenesis is essential before any work on abnormal melanogenesis can be properly designed, carried out and evaluated. The long range aim of this project is the study of melanomas and other types of pigmentary disorders. Since both melanotic and amelanotic melanomas apparently involve aberrant cellular control mechanisms, we feel most strongly that our current research may be the most direct approach to the study and ultimate control of this dread disease.

Proposed Course of Project:

1) The work on the ultrastructural aspects of melanogenesis of the pigment mutants of the mouse is virtually completed. Future work planned involves a similarly approached study on human melanogenesis, both in normal and abnormal conditions.

2) Concerning the biochemistry of melanogenesis, future plans call for more extensive solubilization studies of mature melanin granules, as well as the isolation and characterization of immature granules, e.g. the premelanosomes of the albino. Again, once the biochemical parameters of study of melanogenesis in mice have been established, the scope of study will be expanded to include the study of these granules in normal humans, as well as those in various pathological conditions, including both melanotic and amelanotic melanomas.

3) Control of tyrosinase activity in normal and albino mutants of mice will continue to be investigated. The characteristics of tyrosinase control mechanisms in other mouse pigment mutants, as well as the control of the enzyme in normal and pathological human conditions will be studied.

Honors and Awards: None

Publications:

1) Lutzner, M. A.: Ultrastructure of the development of the normal black and giant beige melanin granules in the mouse. In Riley, V. (Ed.): Pigmentation. New York, New York, Appleton-Century Crofts, 1970. (In Press).

2) Lutzner, M. A.: Ultrastructure of giant melanin granules in beige mutants of the mouse. In Montagna, W. (Ed.): VIII Pigment Cell Conference. 1969. (In Press).

Serial No. NCI-3638
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of DNA repair in Xeroderma Pigmentosum

Previous Serial Number: None

Principal Investigator: Jay H. Robbins, M.D.

Other Investigators: Kenneth H. Kraemer, M.D. William R. Levis, M.D., A. Edgar Miller, M.D.

Cooperating Units: Rosemary Cuddy, Program Analysis and Reporting Section, EA, NCI.

Man Years (July 1, 1971 through June 30, 1972)

Total:	1.8
Professional:	1.2
Other:	0.6

Project Description:

Objectives:

To study the DNA excisional repair process in normal cells and in cells from patients with xeroderma pigmentosum (XP). The role of this process in repair of damage caused by ultraviolet (UV)-radiation, alkylating agents and other chemicals is being evaluated.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: Nature and modification of precancerous state and determination of mechanisms accounting for high degrees of stability of cell functioning.

Material:

Lymphocytes and other cells are obtained from hospitalized patients and outpatients.

Methods Employed:

Normal and XP patients' cells are irradiated with ultraviolet (UV) light and then incubated with radioactive thymidine. The degree of incorporation of radioactive label into the DNA of the cells is determined by

autoradiography and by scintillation counting. 'Purified' lymphocytes are obtained separated from other leukocytes by passage through nylon fiber columns. Fibroblasts are obtained from skin biopsies and cultured in vitro. Epidermal cells are obtained from dermatome shavings and are studied as an intact tissue or dissociated by trypsin. XP and normal cells are irradiated with UV-light or treated with alkylating or other chemicals. The UV-induced incorporation of tritiated thymidine (^{3}HTdR) into DNA is determined.

Major Findings:

The DNA repair defect know to occur in 3 of our 4 original XP patients has been found in lymphocytes of 4 additional patients. Two of these patients have associated neurological complications. Their lymphocytes perform approximately 30-55% of the normal amount of UV-induced ^{3}HTdR incorporation during the first 3 post-irradiation hours and continue to incorporate for a longer time than normal cells until they eventually incorporate as much ^{3}HTdR as normal cells.

The prolonged UV-induced ^{3}HTdR incorporation we originally discovered in XP lymphocytes has now been shown to occur in UV-irradiated cells of each of our 7 XP patients who have an abnormally low rate of incorporation immediately after irradiation. Demonstration of such prolonged incorporation is, therefore, a useful diagnostic feature of the repair defect in XP. This prolonged incorporation has now been shown to be resistant to hydroxyurea (HU), nuclear in localization, and to cease when the XP lymphocytes have incorporated as much ^{3}HTdR as normal lymphocytes. These results suggest, therefore, that these XP cells can perform as much DNA repair as normal cells but require a longer period in which to accomplish this incorporation.

Dose-response curves of ^{3}HTdR incorporation as a function of irradiation have been obtained for XP and normal lymphocytes. The shape of the curves appear generally to be similar.

Studies of UV-induced ^{3}HTdR incorporation into normal and XP epidermal cells are being conducted. The preliminary results indicate that the results with epidermal cells correlate with results from fibroblasts and lymphocytes. UV-induced ^{3}HTdR incorporation is being studied in epidermal cells from our unique XP patient whose lymphocytes and skin fibroblasts have normal DNA repair. Preliminary results indicate that this patient's epidermal cells, the primary clinical site of his disease, have either a normal, or near normal, rate of UV-induced ^{3}HTdR incorporation. These results suggest, therefore, that the malignancies and clinical signs of XP may have more than one etiology or that the repair defect present in the great majority of XP patients may be etiologically unrelated to their disease.

Significance to Bio-medical Research and the Program of the Institute:

UV-radiation is believed to be the major cause of the most common type of human cancer, cancer of the skin. Patients with XP are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have an excisional repair process which rapidly repairs certain types of DNA

damage, while most XP patients have a marked impairment in the rate of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Elucidation of the possible relationship between repair deficiency and skin tumor development in XP patients would, therefore, have wide application to other types of human cancer which may be due to exogenous or endogenous chemical carcinogens.

Proposed Course of Project:

Continuation of research as indicated in the foregoing.

Honors and Awards: None

Publications:

1) Robbins, J. H. and Kraemer, K. H. Abnormal rate and duration of ultraviolet-induced thymidine incorporation into lymphocytes from patients with xeroderma pigmentosum and associated neurological complications. Mutation Research. (In press).

2) Robbins, J. H. and Kraemer, K. H. Prolonged UV-induced thymidine incorporation into xeroderma pigmentosum lymphocytes: studies on its duration, amount, localization and relationship to hydroxyurea. Biochimica Biophysica. (In press).

Serial No. NCI-3639
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Epithelial - Leukocyte Interactions

Previous Serial Number: None

Principal Investigator: William R. Levis, M.D.

Other Investigators: A. Edgar Miller, M.D.

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total: .7
Professional: .6
Other: .1

Project Description:

Objectives

1) To develop a useful and sensitive histocompatibility test in humans.

2) To develop an in vitro model for the study of homograft rejection in humans.

3) To develop an in vitro system for studying immune reactivity to various skin cancers.

4) The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.3: Whole organism control and systemic modifiers of oncogenesis.

Material:

1) Human Peripheral Blood
2) Keratome strips of human skin
3) Excised tumors of human skin

Methods Employed:

Human peripheral blood leukocytes are cultured in vitro as outlined in project "Tissue Culture Studies of Human Lymphocytes." (NCI-3607)

Human epidermal cells are obtained by keratome stripping and subsequent exposure to trypsin.

Various concentrations of leukocytes and epidermal cells are mixed, incubated for 3 to 6 days and then assayed for DNA synthesis.

In future studies neoplastic cells from various skin cancers will be utilized and compared with normal epidermal cells.

Major Findings:

Human epidermal cells induce blastogenesis in allogeneic leukocytes. Human epidermal cells did not induce blastogenesis in autologous leukocytes nor in the leukocytes of a mixed leukocyte HL-A identical sibling pair. These results indicate that epidermal induced leukocyte blastogenesis is a measure of histocompatibility in humans comparable to a one - way mixed leukocyte reaction.

Significance to Bio-Medical Research and the Program of the Institute:

Findings thus far indicate that mixed skin-leukocyte cultures offer a useful histocompatibility test in humans. Furthermore, mixed skin-leukocyte cultures offer a useful *in vitro* model for study of homograft rejection. The techniques employed in these experiments as well as the knowledge of the histocompability reaction have paved the way for studies using contact sensitizers such as DNCB *in vitro*.

Proposed Course of Project:

These studies will facilitate studies on contact dermatitis both in a clinical setting and as an additional tool in studying cellular immunity *in vitro*. Furthermore, the use of normal epidermal cells has paved the way for studies on immune reactivity induced by various skin cancers. These studies are currently being initiated and some are being performed in conjunction with immunotherapy studies on Basal Cell Carcinomas.

Honors and Awards: None

Publications: None

Serial No. NCI-3640
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Use of Methanol fixed paraffin embedded epidermis for detection of Antinuclear Antibodies

Previous Serial Number: None

Principal Investigator: Arthur R. Ugel, M.D.

Other Investigators: Stephen B. Guss, M.D.

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	.5
Professional:	.5
Other:	0

Project Description:

Objectives:

To determine if methanol fixed paraffin embedded epidermis is a suitable substrate for the detection of antinuclear antibodies.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: Nature and modification of precancerous state and determination of mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

Cow hoof epidermal and human heel epidermal sections were fixed in 80% methanol and processed for 16 hours under vacuum by an automatic tissue processor. Sections were dehydrated and cleared of paraffin as needed. Standard immunofluorescent techniques were employed for demonstration of antinuclear antibodies.

Major Findings:

1) Use of paraffin embedded cow hoof allowed a sensitivity equal to that of cryostat sections for detecting A.N.A. Furthermore, sections had a better preservation of tissue architecture, resulting in easier

interpretation.

2) Anyone can now perform the A.N.A. test easily since paraffin embedded sections can be prepared ahead of time or purchased rather than prepared each time one performs the test as must be done with cryostat sections.

Significance to Cancer Research:

This technique should facilitate the immunofluorescent detection of most, if not all, circulating antibodies, including tumor-specific antibodies.

Proposed Course of Project:

Completed

Honors and Awards: None

Publications:

Guss, S. B. and Ugel, A. R.: Paraffin Embedded Epidermis for Antinuclear Antibodies. Arch. Derm. (In press).

Serial No. NCI-3641
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunotherapy of Skin Cancers with DNCB

Previous Serial Number: None

Principal Investigator: William D. Terry, M.D.

Other Investigators: William R. Levis, M.D., Gary L. Peck, M.D., Kenneth H. Kraemer, M.D.

Cooperating Units: Immunology Branch, NCI

Man Years (July 1, 1971 through June 30, 1972)

Total:	.8
Professional:	.8
Other:	0

Project Description:

Objectives :

1) To determine the efficacy of 2, 4 dinitrochlorobenzene (DNCB) on cutaneous malignancies and premalignant growths in subjects sensitized to DNCB.

2) To evaluate DNCB as a useful model for the study of immunotherapy of certain human cancers and possibly lead to additional immunotherapy studies.

3) The Objective and Approach of the National Cancer Plan that this project most closely supports is 6.1: Develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (alone) and in combinations in the cure and palliation of cancer.

Material:

1) Patients with multiple skin cancers
2) 2, 4 Dinitrochlorobenzene
3) acetone
4) acid mantle creme

Methods Employed:

1. Sensitization: Subjects with multiple skin cancers are sensitized by challange with DNCB (2,000 ug in 0.1 ml of acetone or 50 mgms/day of acid mantle creme).

2. Titration of Dose for Application: Serial dilutions of DNCB beginning with 100 ug in 0.1 ml acetone or 50 mgm of the 1:10,000 DNCB in aqueous base are applied as patch tests to 1 cm areas of normal skin 10 to 14 days following sensitization.

3. Methods of Challange to tumors: The initial concentrations of DNCB applied to the tumor surface is 10 fold lower than the lowest concentration which elicits a positive patch test as determined by step 2. The DNCB is applied to the tumor either in acetone or in acid mantle creme. Application will be twice daily for a period of up to eight weeks. Residual lesions will be followed for 4 more weeks. If they do not regress completely, another course of therapy will be undertaken.

4. In all individuals with multiple skin lesions a number of lesions will serve as controls. These lesions will be treated either with acetone alone or with acid mantle creme which does not contain DNCB. Some lesions will not be treated at all and some lesions will be treated only with a primary irritant such as croton oil or Na lauryl sulfate.

Major Findings:

Preliminary results indicate that DNCB has some effect in the treatment of basal cell carcinomas. Three patients had moderate to good partial responses, i.e. an over all decrease in tumor mass. One patient developed tolerance to DNCB and his tumors evidenced no detectable response. Only a small number of the total tumors treated disappeared completely with the longest follow up being 6 months. One tumor enlarged considerably while under treatment and the remainder of the treated tumors partially regressed or remained the same. It is still too early in the study to compile the exact percentage of total and partial regressions as it is felt at least a one year follow up is required to assess regression and progression on the generally slow growing basal cell carcinomas.

Significance to Bio-Medical Research and the Program of the Institute:

Contact sensitizers such as DNCB produce a delayed type hypersensitivity reaction. Delayed or cellular immunity appears to be an important type of host immunity against tumor in man as well as other mammals. The mechanism whereby DNCB induced repression of basal cell carcinomas is unknown but is probably related to the delayed type hypersensitivity provoked around, on and in the challanged tumor. The use of DNCB and possible other contact sensitizers offers a tool to control cellular immunity around cutaneous malignancies and to ultimately eradicate these tumors.

Proposed Course of Project:

In the future, it might be possible to extend the principles learned from immunotherapy of cutaneous malignancies to a variety of other less accessible malignancies. However, the immediate course of this project will be to continue using DNCB on more patients, varying the concentration and scheduling of the DNCB and control solutions, and ultimately attempting other contact sensitizers such as Pentadecacatechol.

Honors and Awards: None

Publications: None

Serial No. NCI-3642
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Treatment of Psoriasis with Topical Vitamin A Acid

Previous Serial Number: None

Principal Investigator: Gary L. Peck, M.D.

Other Investigators: Douglas J. Key, M.D. and Stephen B. Guss, M.D.

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	.15
Professional:	.15
Other:	0

Project Description:

Objectives:

1) To evaluate safety and effectiveness of topical Vitamin A Acid in psoriatic patients.

2) The Objective and Approach of the National Cancer Plan that this project most closely supports is 6.1: Develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (alone) and in combination in the cure and palliation of cancer.

Agents:

0.05% to 0.3% Vitamin A Acid (Eastman) in unibase

Material:

Adult patients with psoriasis. Females must be past the childbearing age.

Methods Employed:

1) Patients must have psoriasis previously unresponsive to standard therapy.

2) They must have normal kidney, liver, and bone marrow function.

3) Medication will be applied twice daily until clear.

4) Patients will avoid sun exposure to prevent phototoxicity, if any.

5) Laboratory tests measuring bone marrow, liver, and kidney function will be obtained before, during, and 6 weeks after cessation of therapy.

6) Serial photographs will be obtained.

Major Findings:

1) No preferential beneficial response was seen in the treatment of 14 psoriatic patients using Vitamin A Acid cream without plastic occlusion either (1) at a concentration of 0.05% for 12 weeks in a double blind manner against unibase control, or (2) at concentrations varying from 0.1 to 0.3% for 6 weeks at any given dosage level in comparison with a corticosteroid cream.

2) Three of nine patients treated with 0.25% Vitamin A Acid cream under plastic occlusion in a double blind manner showed definite evidence of clearing limited to Vitamin A Acid treated areas. This response, which was just evident 2 to 4 weeks after onset of therapy, was short-lived in two of these patients, who also failed to respond to subsequent courses of therapy.

Significance to Cancer Research:

1) This study is part of an ongoing search for an effective topical therapy for psoriasis.

2) It is hoped that this study will contribute to the pharmacology of chemotherapeutic agents.

Proposed Course of Project:

1) Almost concluded.

2) In light of reports claiming beneficial results in the treatment of psoriasis with topical Vitamin A Acid, we may repeat this study using Vitamin A Acid obtained from another source (Roche) and study the effect of using different inert vehicles for the active drug.

Honors and Awards: None

Publications: None

Serial No. NCI-3643
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Wound Healing in Darier's Disease (Keratosis Follicalaris)

Previous Serial Number: None

Principal Investigator: Gary L. Peck, M.D.

Other Investigators: Peter M. Elias, M.D.

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	.15
Professional:	.15
Other:	0

Project Description:

Objectives:

1) To determine if the accelerated healing of dermatome stripped diseased skin in Darier's disease is due to an accelerated epidermal migration.

2) To study the effect of experimental wounding on the clinical course of both involved and uninvolved skin.

3) The Objective and Approach of the National Cancer Plan that this project most closely supports is 4.1: Biology of cells capable of forming cancers - steps preceding cancer development at cell transformation (and other) body sites: determine rates of (cancer) cell transformation and percent of rakes leading to tumor formation.

Material:

Normal and diseased skin in a patient with Darier's Disease

Methods Employed:

Dermatome Stripping. Strips of skin were taken from a 62-year-old female patient with Darier's disease using a Castroviejo ophthalmologic keratome (Storz Instrument Co.). Strips were approximately 2 cm. in width, 5 cm. in length and 0.2 mm. thick, determined by using a 0.2 mm. spacer in the

shaving head. Sites included several areas of the back, upper abdomen and hips. Bleeding was controlled with pressure only. Telfa gauze dressings were used for one to two days, and no other topical therapy was applied. The wounds were then left open, measured and photographed daily.

Incisional Wound Healing. Multiple linear incisions, approximately one cm. in length, were made in diseased skin areas of the right anterior forearm and in clinically uninvolved skin of the left anterior forearm. Because the involved skin surface is more irregular than uninvolved skin, and because the epidermis is variably thickened in dermatoses such as Darier's disease, the standard blade guard device can not be used to ensure equal depth of incisions. We, therefore, chose minimal capillary bleeding as a measure of comparability of incision depth. Telfa gauze was the only dressing employed. Four mm. punch biopsies were taken at zero time, and 3, 6, 12, 18, 24, 48 and 72 hours after wounding. Each biopsy was divided transversely to the wound into two approximately equal pieces. One piece was fixed in cold phosphate-buffered glutaraldehyde, post-fixed in osmium tetroxide, imbedded in Epon 812, and 1-2 μ thick sections were stained with Azure II-methylene blue. The other half was routinely fixed in formaldehyde and imbedded in paraffin prior to sectioning and staining. Sections were cut coronally to the incision.

Major Findings:

1) Dermatome Stripping. Involved sites healed more rapidly than uninvolved skin following dermatome stripping. Two weeks after stripping, the uninvolved site remained larger and more erythematous than the involved site. Furthermore, a zone approximately 10 cm. wide of previously diseased skin around stripped sites on the left hip was observed to have clinically regressed by this time. Clinical regression of involved skin surrounding dermatome stripped sites was repeatedly observed.

   Five months after the initial stripping experiment, the patient had a generalized exacerbation of her disease, which spared previously dermatomed sites of uninvolved skin. Clinically, the uninvolved stripped sites are now hypopigmented and slightly atrophic. Histologically, the epidermis and pilosebaceous apparatus appear normal except for diminution of the rete ridge pattern and flattening of the papillary dermis.

2) Incisional Wound Healing. Superficial linear incisions healed more quickly in diseased sites (12 hours) than in uninvolved sites (48 hours) indicating accelerated epidermal migration.

Sifnificance to Cancer Research:

Knowledge of the controls governing cell migration after injury are essential to the understanding of malignant processes.

Proposed Course of Project:

1) Ultrastructural examination of the above material.

2) Confirmation of these findings in other patients with Darier's disease.

Honors and Awards: None

Publications:

Peck, G. L., M.D. and Elias, P. M., M.D.: Wound Healing in a Patient with Darier's Disease: Preliminary Observations. In Maibach, H. I. and Rovee, D. (Eds.): Epidermal Wound Healing. Chicago, Ill., Year Book Medical Publishers, 1972, pp. 357-363.

Serial No. NCI-3644
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Establishment of a Screening System for Topical Chemotherapeutic Agents in the Therapy of Skin Cancer, Mycosis Fungoides, and Psoriasis

Previous Serial Number: None

Principal Investigator: Gary L. Peck, M.D.

Other Investigators: Eugene J. Van Scott, M.D., Howard I. Maibach, M.D., Phillip Frost, M.D., Gerald Weinstein, M.D., Irwin Freedberg, M.D., Howard Baden, M.D. and Henry Roenigk, M.D.

Cooperating Units:

Temple University, University of California Medical Center (San Francisco), University of Miami - Mt. Sinai Hospital, University of Miami, Boston Beth Israel Hospital, Massachusetts General Hospital and Cleveland Clinic.

Man Years (July 1, 1971 through June 30, 1972)

Total:	.15
Professional:	.15
Other:	0

Project Description:

Objectives:

1) The establishment of a scientific program in which present and future chemotherapeutic agents may be screened topically for antimitotic potential, toxicity, and possible therapeutic use initially in malignant skin diseases, such as cutaneous carcinoma and mycosis fungoides, and subsequently in benign hyperplastic diseases such as psoriasis.

2) The Objective and Approach of the National Cancer Plan that this project most closely supports is 6.2: Determine new sources of chemical agents new means of their selection and additional screens for their selection.

Patients Needed:

At least 10 adult men or women for each chemotherapeutic agent tested

topically in the treatment of skin cancer, mycosis fungoides or psoriasis. Females must be past the childbearing age.

Methods Employed:

After chemotherapeutic agents have been supplied by the Chemotherapy Branch, NCI, and after these agents have been screened for biological activity and safety in experimental models, then large scale clinical testing will be done.

The clinical protocols of this large scale clinical testing have not been formulated as yet. However, the basic design will be similar to NCI-3631 and NCI-3633.

Major Findings:

In progress.

Significance to Cancer Research:

1) This program is a national cooperative study designed to discover superior topical chemotherapeutic agents than those which are currently available in the treatment of skin cancer, mycosis fungoides, and psoriasis.

2) We would also hope to contribute to the knowledge of the clinical pharmacology of these agents. Skin cancer should prove to be a valuable lesion for the screening of this and future topical chemotherapeutic agents because the tumors are observable, measurable, and therefore, can be used as an objective indicator of response to therapy. Furthermore, the patients can be expected to be in good health, which would allow for long term studies.

Proposed Course of Project:

The entire design of this screening system, including preclinical screens and large scale clinical testing, is currently being established with the assistance of the associate investigators and a committee of pharmacologists, toxicologists, and chemotherapists. Merger of this program with a similar one in the Arthritis Institute is anticipated.

Honors and Awards: None

Publications: None

Serial No. NCI-3645
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Porokeratosis Plantaris, Palmaris, et Disseminata

Previous Serial Number: None

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Stephen B. Guss M.D.

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:	.01
Professional:	.01
Other:	0

Project Description:

Objectives:

To examine a kindred of which eight members had multiple, superficial porokeratoses.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 5.2: Identification of precancerous lesions and other states associated with cancer.

Methods Employed:

This report concerns eight patients in four generations of a single family with multiple porokeratoses. We examined five of these eight patients. Three of these five had skin punch biopsies. The other three patients with PPPD were contacted indirectly and had their clinical diagnosis verified by history and photographs. In addition, ten other members of the family were examined and 30 were contacted indirectly.

Major Findings:

Eight members from four generations of a family had multiple superficial porokeratoses on the trunk, palms, and soles. This appears to be a third type of porokeratosis, distinct from the classic Mibelli type or

the Chernosky disseminated superficial actinic porokeratosis (DSAP) type. The disease begins in the late teens and early twenties, is transmitted as an autosomal dominant, and affects males twice as often as females. The hallmark of this disease is the early appearance of plantar and palmar lesions with subsequent involvement of other areas of the body including surfaces not exposed to ultraviolet radiation. Morphologically the clinical lesions resemble Chernosky's DSAP. Histologically it has the identifying features of porokeratosis with a cornoid lamella. One of our eight patients developed squamous cell carcinomas in areas of porokeratosis. No effective mode of therapy has been found.

Significance to Cancer Research:

This is the first description of an autosomal dominant, probably precancerous, genodermatosis.

Proposed Course of Project:

Concluded

Honors and Awards: None

Publications:

Guss, S. B., M.D., Osbourn, R. A., M.D., and Lutzner, M. A., M.D.: Porokeratosis Plantaris, Palmaris, et Disseminata. Arch. Derm. 104: 366-373, 1971.

Serial No. NCI-3646
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Dermatome Shaving in the Treatment of Xeroderma Pigmentosum

Previous Serial Number: None

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Ervin H. Epstein, Jr., M.D., Peter G. Burk, M.D., I. Kelman Cohen, M.D., and Peter Deckers, M.D.

Cooperating Units: Surgery Branch NCI

Man Years (July 1, 1971 through June 30, 1972)

Total:	.01
Professional:	.01
Other:	0

Project Description:

Objectives:

To study the effect of dermatome shaving in a patient with xeroderma pigmentosum.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 6.1: Develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (alone) and in combinations in the cure and palliation of cancer.

Material:

One patient with xeroderma pigmentosum

Methods Employed:

Split thickness strips, approximately 0.017 inch thick, were removed with an electric dermatome from previously untreated areas of both legs.

Major Findings:

In this patient with XDP no tumors have developed in areas of the face that were dermabraded seven years ago. The results of facial dermabra-

sion in this patient are cosmetically better than those depicted in a recent case report advocating resurfacing with full-face skin grafts taken from less damaged areas.

Significance to Cancer Research:

Dermatome shaving appears to be a new, useful technique in the treatment of XDP, resulting in the retardation of malignant tumor development and in a cosmetically more normal appearance.

Proposed Course of Project:

Terminated

Honors and Awards: None

Publications:

Epstein, E. H. Jr., M.D., Burk, P. G., M.D., Cohen I. K., M.D., Deckers P., M.D.: Dermatome Shaving in the Treatment of Xeroderma Pigmentosum. Arch. Derm. 105: 589, 590, 1972.

Serial No. NCI-3647
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Fine structural and cytochemical studies of the cell biology of granular leukocytes and skin, including: 1) the origin, nature, and fate of the characteristic cytoplasmic granules of neutrophils, eosinophils, and basophils; 2) the application of scanning electron microscopy (SEM) in the study of skin, blood cells, viruses, and other soft biological tissues; 3) the application of transmission electron microscopy (TEM) and cytochemistry in studies of skin and related tissues.

Previous Serial Number: None

Principal Investigator: Bruce Wetzel, Ph.D.

Other Investigators: Joan M. Bull, M.D., Seymor H. Wollman, M.D., Robert Buscho, M.D., Raphael Dolin, M.D., Peter Elias, M.D., William R. Levis, M.D., Gary L. Peck, M.D. and Reuben F. Gittes, M.D.

Cooperating Units: Medicine Branch, NCI; Laboratory of Physiology, NCI; Laboratory of Infectious Diseases, NIAID; Department of Urology, University Hospital, San Diego, California

Man Years (July 1, 1971 through June 30, 1972)

Total:	4.0
Professional:	1.0
Other:	3.0

Project Description:

Objectives:

1. To characterize definitively the mechanisms of granulogenesis in leukocytes, to determine the contents of these granules, and to clarify the fate and function of normal and diseased granulocytes and their organelles.

2. To develop methods and interpretative criteria for the application of SEM to soft biological tissues, and thereby to elucidate the structure of normal, diseased, and experimentally altered cells, and viruses in skin, peripheral blood, and other sites.

3. To apply TEM and cytochemistry in studies of skin related tissues, as well as in other studies of basic biological or methodological interest.

4. The Objective and Approach of the National Cancer Plan that this project most closely supports is 4.2: Biology of cells capable for forming cancer and interactions which host cells - precancerous metabolic and morphologic lesions.

Methods Employed:

1. Granular leukocytes from bone marrow, peripheral blood, peritoneal exudates, tissue culture, and sites of tissue infiltration are processed by a wide range of methods for TEM and cytochemistry including various fixatives, stains, and techniques for enzyme localization. Experiments are in progress to adapt critical autoradiographic techniques at both light and electron microscope levels to quantitative studies of radioisotope incorporation into protein and mucosaccharides of the characteristic granules of these cells. Tissues from rabbits, guinea pigs, rats, mice, and humans are under investigation.

2. A variety of preparative methods for SEM are being explored and compared in several types of soft tissue. Specifically, fresh or in vitro specimens are treated with aldehydes or metal salt fixatives, dessicated by one of several critical point procedures or by freeze-drying, mounted with various substrates, and examined under a wide range of SEM operating parameters (viz., accelerating voltage, beam current, working distance, and signal collection mode) in order to define the optimal conditions and the limitations of this relatively new approach.

   Tissues examined thus far include explants from human fetal intestine in organ culture with and without infection by known viral pathogens, human buffy coat cells in vitro, with and without mitogens, embryonic chick skin in organ culture, with and without additional Vitamin A, and freshly excised rat thyroid glands after thyrotropin stimulation. Parallel specimens of most of these tissues are also viewed by TEM or light microscopy.

Major Findings:

1. Modifications in our routine fixation procedures for TEM (e.g., exposure to 2% tannic acid between aldehyde and osmium tetroxide fixation) have revealed granules in rabbit neutrophils which resemble a class of tertiary granules previously indistinguishable on a morphological basis in aldehyde-fixed cells and thus overlooked or ignored by many workers.

   Fine structural studies of cultured cells from human bone marrow have provided further evidence for the growth and differentation of neutrophil and eosinophil lines in such preparations, although the cells are atypical and seem to transform into a common sort of macrophage

in older cultures.

A comparative study of the effects of various fixatives on developing granulocytes in rabbit marrow has resolved a serious discrepancy in the literature in which early stages in neutrophil development have been interpreted as a special form of early eosinophil; eosiniphil granules are much more resistant to extraction and other artifact than neutrophil granules, and can thus be readily distinguished in specimens where the neutrophils are consistently altered by the fixative.

2. An effective and reliable method which employs glutaraldehyde fixation and critical point drying with CO_2 has been chosen and modified for the preparation of soft biological tissues for SEM. With this approach, we are obtaining new information in a number of studies undertaken to develop and test our methods and our interpretation of SEM results.

   Specifically, extensive areas of epithelial surface with well preserved microvilli can easily be surveyed in the cultured intestinal explants by this method. Small efflorescences of elongated microvilli have been seen in all such specimens and their presence confirmed in parallel specimens examined by TEM and in lateral views of deliberately fractured villi. Such fractures expose the lateral membranes of the epithelial cells, and these are marked by prominent curvilinear ridges which probably correspond to gap junctions seen by TEM. After five days in culture, the villi tend to become shorter and broader, some individual epithelial cells swell to form small surface blisters, and the lateral intercellular spaces between epithelial cells sometimes appear swollen.

   Cultured leukocytes from human buffy coats display distinctive surface structures which presumably reflect adherence, locomotion, cellular interaction, transformation into blastoid forms, and cellular degeneration. Large broad membranous processes which initially characterize the macrophages in these preparations are reduced or absent from many cells after an hour in vitro, and at least some of these cells flatten and spread on the substrate. Extraordinarily long (up to 100 μm) tenous, unsupported processes have been observed between leukocytes after 15 minutes in the presence of phytohemagglutinin, and in later cultures the characteristic association of lymphocytes and macrophages in this system is well displayed.

   The microvilli on the luminal surfaces of rat thyroid follicles vary in length among different follicles and sometimes from cell to cell, and their distribution is not entirely uniform over the cell surface. The pseudopods which engulf luminal colloid in response to TSH stimulation can be characterized and counted much more efficiently and definitively than in sectional material, and their three-dimensional morphology during formation and regression is under investigation.

Specimens of embryo chick skin cultured with additional Vitamin A display mucous metaplasia with characteristic changes in the growth pattern and epithelial cell surface; clusters of round epithelial cells accumulate beneath the embryonic periderm, and the regular surface of these cells alters to an irregular array of rather long microvilli. The nature and extent of these changes is readily appreciated in rather large samples which can easily be surveyed by SEM.

3. A manuscript reporting prior work on the hyperplasia and hypertrophy of thyroid parafollicular ("C") cells in rats made hypocalcemic by parathyroidectomy has been revised for publication.

Significance to Cancer Research:

1. The characteristic granules of neutrophils, eosinophils and basophils are the principal indices of differentiation and the principal effector organelles in these cells; a thorough understanding of the origin and nature of these granules is necessary to define and interpret the differentiation of normal granulocytes, the cytopathology of chronic granulocytic leukemia and related disorders, and to help clarify the nature and functions of granulocytic infiltrates in skin and other tissues in malignant diseases.

2. SEM reveals fine surface structure with unparalled three-dimensionality, depth of field, and sampling capability; as this approach is adapted to biological problems, cellular form, surface details and interactions can be examined in normal and malignant tissues, as well as in experimental systems which help define immune responses, differentiation, and other processes which are fundamental to oncogenesis and its control. The study of viral infection in intestinal explants should suggest new approaches to problems in viral oncology with this technique; the study of buffy coat cells in culture offers a novel and informative view of cells undergoing blastogenesis and can serve as a basis for comparison in SEM of leukemic cells; the further delineation of normal thyroid form and function bears on our understanding of hormonal balance in health and disease and provides further basis for comparison with tumors of this gland; and the studies of embryonic chick skin provide more information on the process of differentiation in this tissue.

Proposed Course of Project:

1. As stated under Project Description, with major emphasis on quantitative autoradiography. Light microscopic monitoring of fixation, processing, and culture conditions in thin perfusion chambers is also planned.

2. To further refine techniques of specimen preparation for SEM and to explore other modes of SEM for novel cytochemical methods (e.g. cathodoluminescent stains). To study the effects of a number of known viral pathogens on human intestinal explants in organ culture with these methods. To study leukocytes under the conditions noted above, to

compare leukocytes from other normal sources (lymph nodes, thymus, marrow, etc.) and from certain leukemias. To complete the SEM studies of chick skin and thyroid as noted above.

3. To engage in collaborative fine structural and cytochemical studies of skin and other tissues in order to sustain a broad expertise in basic cell biology, and to acquaint myself more with the special problems of skin cytology.

Honors and Awards: None

Publications:

1. Wetzel, B. K., Buscho, R., and Dolin R.: Scanning microscopy of human intestinal explants in organ culture. Proceedings of the 30th Meeting of Electron Microscopy Society of America. (In press).

2. Wetzel, B. K., Gittes, R. F.: Thyroid light cell hyperplasia in rats with chronic hypocalcemia. Zeitschrift fur Zellforschung und Mikroskopische Anatomie. (In press).

Serial No. NCI-3648
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of Structure and Function of Abnormal Cells in the Sezary Syndrome, Mycosis Fungoides and Related Lymphomas

Previous Serial Number: NCI-3617 (Part of)

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: None

Cooperating Units: M. Prunieras, Laboratoire de Recherches, sur les Tumeurs de la Peau Humaine, Fondation Ad. de Rothschild, 29 rue Manin, Paris 19e, France; I. Emerit, Laboratoire de Cytogenetique, Faculte de Medecine, Paris Vie, France; G. Flandrin, Laboratoire de Cytologie, Institut de Recherches sur les Leucemies, Hopital St Louis, Paris 10e, France; C. Grupper and R. Durepaire, Service de Dermatologie, Foundation Ad. de Rothschild, 29 rue Manin Dermatologie, Foundation Ad. de Rothschild, 29 rue Manin, Paris 19e, France.

Man Years (July 1, 1971 through June 30, 1972)

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

Objectives:

To study structure, function and oncological significance of the characteristic cell of the Sezary syndrome, and to study similar parameters in malignant cells of mycosis fungoides and related cancers.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 4.2: Biology of cells capable for forming cancer and interactions which host cells - precancerous metabolic and morphologic lesions.

Methods Employed:

1. Patients sought with Sezary syndrome and with mycosis fungoides.

Specimens of blood, marrow, skin node, etc. will be obtained for study.

2. Tissue cells and buffy coat preparations will be Feulgen-stained and examined with a cytophotometer in order to detect presence of polyploid cells.

3. Tissue biopsies and buffy coat pellets will be examined with the electron microscope for morphologic identification of characteristic cells.

4. Chromosome analysis of abnormal tissue, marrow and blood cells will be made in order to determine if chromosomes are normal or abnormal in number and structure.

5. Immunologic techniques will be used to determine whether Sezary cells belong to either B- or T-lymphocyte class.

Major Findings:

1. Three patients with Sezary syndrome have been identified by finding typical cells in blood with electron microscope. All 3 patients had polyploid cells in peripheral blood as identified by cytophotometry. All 3 patients had blood cells in culture with abnormal chromosome number. In addition, 2 of the 3 had "marker" chromosomes. Mitosis occurs in culture only with addition of PHA, indicating lymphocyte nature of cells. Immunological determinations show Sezary cells to be devoid of immunoglobulin at cell surface, indication of possible T-cell origin. Also, 2 patients with typical mycosis fungoides, have been studied cytophotometrically and cytogenetically and preliminary results show presence of polyploid cells. No marker chromosomes have yet been found. Other patients are being studied.

   Finally, 2 patients, at first considered to have Sezary syndrome, are now thought to be different: (1) the cells have abnormal nuclei but not as convolted as Sezary cells, (2) the cells are smaller than abnormal cells of Sezary syndrome, (3) Cytophotometry is normal, (4) chromosome markers are present but chromosome number is pseudodiploid or hyperdiploid rather than near tetraploid as in Sezary syndrome.

Significance to Cancer Research:

Chronic lymphocyte leukemia has been recently been found to be a B-lymphocyte malignancy. Evidence is accumulating that Sezary syndrome may be a T-lymphocyte malignancy. If this is so, different approaches, both chemotherapeutic and immunological might be taken with this syndrome.

Proposed Course of Project:

To study additional patients and continued cytogenetic and immunologic investigations. To study additional parameters of structure and function in an attempt to improve diagnosis of this form of cancer and to gain further insight into the biology of these unusual cancer cells and the etiology of their malignant transformation.

Honors and Awards: None

Publications: None

SUMMARY OUTLINE
Annual Report of the Immunology Branch, National Cancer Institute
July 1, 1971 through June 30, 1972

I. IMMUNOCHEMISTRY

A. Cell Surface Antigens and Receptors
B. Immunoglobulins and Related Proteins

II. IMMUNOBIOLOGY OF LYMPHOID CELLS

A. Mechanism by Which Lymphoid Cells Destroy Target Cells
B. Control Mechanism in Growth and Differentiation of Lymphoid Cells and Early Activation Events

III. TRANSPLANTATION IMMUNOLOGY

IV. IMMUNODIAGNOSIS

V. IMMUNOPROPHYLAXIS AND IMMUNOTHERAPY

SUMMARY

Annual Report of the Immunology Branch, National Cancer Institute
July 1, 1971 through June 30, 1972

INTRODUCTION

Of fundamental importance to the field of tumor immunobiology has been the unequivocal demonstration that tumor cells carry on their surfaces antigens which are qualitatively and/or quantitatively different from those present on normal cells. The program of the Immunology Branch is designed to investigate what these antigens signify as regards the tumor cell, what their implications are for the immune system that recognizes them, and how these various factors bear on the applied areas of immunodiagnosis, immunoprophylaxis, and immunotherapy.

I. IMMUNOCHEMISTRY

A. Cell Surface Antigens and Receptors. Appreciation of any derangement of the surfaces of malignant cells will depend upon an understanding of normal cell surfaces. Little is known about the structure of cell membranes, although membrane models have been constructed on the basis of thermodynamic considerations. A major protein constituent of red cell membranes has been isolated and almost totally sequenced (5019). The results provide the first structural evidence supporting a lipid bilayer model with intercalated proteins containing a hydrophobic portion buried in the lipid bilayer and a hydrophilic portion exposed to the cell surface.

Histocompatability antigens are normal cell surface constituents of unknown structure. Their great importance as recognition signals for cell-cell interaction, as well as their possible role in the genetic control of immune responsiveness makes their structural analysis of great importance (5006, 5018).

Receptor sites for mitogens provide other definable cell surface moieties that can be structurally analyzed (5018).

Carcinoembryonic antigen is a tumor associated antigen which can be obtained in reasonable quantity. It has been partially purified and preliminary chemical analyses carried out (5018). The partial amino acid sequence obtained provides the first sequence information available for any tumor associated antigen (5019).

Antigenic analysis of blast cells from acute lymphocytic leukemia has revealed an apparently leukemia specific cell surface antigen (5006). A similar antigen has been found on the surface of human, but not murine, cells after they have been infected with murine leukemia virus. The implications of these observations for the etiology and therapy of leukemia are being pursued.

Differentiation antigens, such as the θ antigen on mouse lymphoid cells, are of great interest because of the opportunity they offer for probing the functional heterogeneity of lymphoid cell populations. This type of differentiation antigen has not previously been described on human lymphoid cells.

Preliminary results of work done with a heterologous antiserum to human fetal thymus cells suggests that a thymus associated antigen does exist on human cells (5019). The functional capacities of cells bearing these antigens will be studied, and attempts will be made to develop a B cell reagent as well.

In vitro assays which permit recognition of cell surface antigens are needed in order that isolation of membrane components can be monitored. A new assay based upon stimulation of protein synthesis (SPS) of lymphoid cells has been devised (5014). This assay permits rapid determination of cellular immunity in mice and guinea pigs and is capable of detecting minor, as well as major, histocompatability antigens. It is currently being evaluated for usefulness in the detection of soluble membrane components.

The biosynthesis of sialic acid and its role in the antigenic structure of lymphoid cell surfaces has been investigated (5015). Removal of sialic acid from normal human peripheral lymphocytes renders them reactive with a "natural" antibody present in autologous serum, thus raising the possibility that this is a normal clearance mechanism for cells with altered cell surfaces.

B. Immunoglobulins and Related Proteins. A complete understanding of the mechanism of antibody-antigen interaction may be important in achieving control of the humoral responses of patients to tumor antigens. Studies of immunoglobulins have provided a three-dimensional model of an IgG molecule at 6A resolution (5002).

Amyloidosis is a disease frequently associated with certain malignancies, as well as with disorders of the immune system. Immunochemical and chemical studies have revealed two classes of amyloid: one having an amino acid sequence identical with that of light polypeptide chains; and one with a sequence unrelated to known immunoglobulins. The pathogenetic implications of these findings are under investigation.

II. IMMUNOBIOLOGY OF LYMPHOID CELLS

A. Mechanisms by Which Lymphoid Cells Destroy Target Cells. One of the lymphoid cells that appears to be important in immune responses to tumors is the effector cell which can kill target cells directly without any evidence of humoral factor involvement. Failure of tumor bearing hosts to reject tumors could reflect qualitative or quantitative inadequacy of this response. Preliminary evidence suggests there is a block in maturation of the effector cell response in tumor bearing animals (5003). In addition, it appears that the antigenic material released by dead tumor cells stimulates humoral, but not cellular, immunity. These observations are being pursued to determine the nature of the maturation block and the role of soluble tumor antigen in tumor bearing animals.

Another mechanism for killing target cells involves lymphocyte dependent antibody (LDA). Target cells coated with this as yet poorly defined immunoglobulin are rendered susceptible to killing by normal (nonimmune) lymphocytes. Heterologous species will make antisera containing LDA, thus raising the

possibility that LDA directed against tumor specific antigens might be able to sensitize tumors for destruction by lymphoid cells (5016, 5006).

B. Control Mechanism in Growth and Differentiation of Lymphoid Cells and Early Activation Events. Studies have been performed to define conditions under which cells can be maintained in prolonged stationary phase in vitro, while maintaining high viability. The nature of the control mechanisms involved have been investigated (5004).

The SPS assay has been used with a double label technique to assess events occurring shortly after stimulation of lymphoid cells with mitogens or antigens (5014). Alterations in protein synthesis have been detected within minutes after stimulation of such cells, and an attempt has been made to characterize the proteins involved.

III. TRANSPLANTATION IMMUNOLOGY

Attempts at marrow grafting of aplastic anemic patients with allogeneic marrow from histocompatable siblings has not been successful in our hands. Graft versus host disease has been a complication of this maneuver (5008). It seems possible, on the basis of experimental work in rodents, that marrow grafting may play a role in the immunotherapy of human malignancy. A preclinical model for the study of graft versus host disease in nonhuman primates has been established (5011). This has necessitated the establishment of reagents for histocompatability typing of rhesus monkeys. Reagents have been prepared and a typing system is operative.

The biologic significance of human histocompatability antigens as they relate to disease susceptibility has been investigated, and strong correlations between certain diseases and specific HL-A antigens have been establishe (5017). The implications of this in terms of the immune response gene system of mice and guinea pigs is being evaluated.

IV. IMMUNODIAGNOSIS

Immunology offers the best hope for techniques appropriate to the improve diagnosis of cancer. A radioimmunoassay has been adapted for carcinoembryonic antigen (CEA) testing and will be utilized to evaluate 1) the possibility that CEA testing could provide an early diagnosis for some cancers and thus favorab alter mortality, and 2) the usefulness of this test in monitoring the effects of therapy in providing an indication of when therapy should be altered (5001)

V. IMMUNOPROPHYLAXIS AND IMMUNOTHERAPY

Immunoprophylaxis experiments have been carried out with BCG in mice developing methylcholanthrene induced tumors (5020). Small positive effects were noted. An immunotherapy experiment using "deblocking" serum is in progress. A program involving immunotherapy of human tumors will be initiated in collaboration with the Surgery Branch, GL&C, NCI, in the near future.

It is intended to continue the work in the five major program areas indicated above, with the intent of maintaining a strong program in fundamental immunobiology coupled with applications in the areas of immunodiagnosis, immunoprophylaxis, and immunotherapy.

Serial No. NCI-5001 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: NCI Immunoglobulin Reference Center

Previous Serial Number: Same

Principal Investigator: William D. Terry, M. D.

Other Investigators: Elaine Esber, M. D.

Cooperating Units: Michael Balese, M. D., Metabolism Branch, NCI

Man Years:

Total:	1
Professional:	1
Others:	0

Project Description:

Objectives: The long range objectives of this project are to find and apply serum diagnostic tests for neoplasma in man. The immediate goals are to establish an NCI Immunoglobulin Reference Center in order to (1) improve immunochemical tests for multiple myeloma and macroglobulinemia by increasing the quality and availability of reagents, techniques and interpretation experience, (2) to facilitate studies of immunoglobulins by establishing national reference standards and by making available to qualified investigators small amounts of reagents that are not commercially available, (3) to investigate the relevance of serum immunoglobulin changes to disease prognosis and response to therapy, (4) to assess myeloma serums for the capacity to bind various well defined antigens, (5) to determine human immune responsiveness of patients with malignant diseases and (6) to purify carcinoembryonic antigens, prepare antisera specific for their detection and to employ these reagents to evaluate the usefulness of CEA tests for the early diagnosis of cancer.

Methods Employed: Immunoelectrophoresis, employing a battery of specific antiserums, is used to detect specific immunoglobulin abnormalities. Quantitative immunochemical techniques, principally radial diffusion in agar plates containing antibody, are used to measure the serum levels of IgG, IgA, IgM and IgD as well as type K and type L immunoglobulins. Protein purification procedures (see Project NCI-5000 1970-1971) are used

to prepare immunoglobulins for use as antigens to induce potent antiserums and as absorbents to obtain specific antiserums. Antiserums are produced in rabbits and larger animals. A modified Farr test using radiolabeled antigens is used to screen all sera for binding activity. Double antibody assays are used for the detection of carcinoembryonic antigen.

Major Findings: The principal effort on this project is conducted through contract PH-43-66-3. The NCI Immunoglobulin Reference Center has been established at Mel-Labs, Inc., in Springfield, Virginia.

Number of serums and urines tested 4/1/71 to 3/31/72	1,000
Amount of specific antisera to human protein supplied to investigators	700 ml
Number of quantitative determinations of Ig concentrations	1,300
Number of antibody assays	1,000

Large volumes of seven different antiserums are required for the diagnostic and quantitative studies. The Reference Center has immunized both small and large animals, collected the antiserums, evaluated them for strength and specificity and, where necessary, has absorbed them with appropriate sera or purified immunoglobulin preparations to render them useful for the immunodiagnostic work.

Immunoglobulin standards are needed, both at the Reference Center and at other institutions doing quantitative immunoglobulin studies throughout the country.

In collaboration with the World Health Organization Immunoglobulin Reference Center, pools of lyophilized plasma have been calibrated with purified proteins, and their stability characteristics evaluated. This lyophilized reference preparation is now available for distribution to any interested laboratory. The collaborative study to accurately calibrate the immunoglobulin content of the reference preparation is underway.

Four hundred sera shown to contain anomalous immunoglobulins have been tested for their ability to bind one of a number of radiolabeled haptenes, such as DNP-lysine. Sixteen sera containing M-proteins demonstrated significant binding activity for one or more of the haptenes. These sera are being investigated to determine whether it is the M-protein that has the binding activity.

A double antibody assay for carcinoembryonic antigen testing has been established. Approximately 800 sera have been tested to validate the assay

which can be performed on 0.2 ml of serum. Materials have been obtained to begin isolation of CEA antigen from tumor tissue.

Significance to Biomedical Research and the Program of the Institute: Clinicians concerned with diagnosis and care of patients and clinical pathologists concerned with laboratory testing have shown great interest in the specific immunochemical (immunoelectrophoresis) tests suitable for screening serum and urine for specific changes characteristic of multiple myeloma and Waldenström's macroglobulinemia. The tests eventually can be carried out throughout the country, but proper performance is required. The Immunoglobulin Reference Center has begun to provide a focus for materials and techniques.

The development of easily performed quantitative tests for immunoglobulins is accelerating research into the effects of disease, chemical therapy and other events on the individual components of the immunoglobulin system. The standardized procedures and high quality reagents at the NCI Immunoglobulin Reference Center provide reference materials and standards for immunoglobulin investigations in the United States.

Evaluation of the possible role of CEA tests in the early diagnosis of cancer has assumed major importance within this contract. This is directly relevant to the institute program directed toward improved diagnosis of cancer.

This project supports Objective 5, Approach 4.

Proposed Course of Project: Major emphasis will be placed upon evaluation of the test for carcinoembryonic antigen. Attempts will be made to define the false positive rate in individuals not known to have malignant disease, in preparation to possible application of this test in a prospective study of a "normal" population. Studies will be initiated to monitor the results of chemotherapy on the level of CEA and to determine whether the test can be used to make therapy more effective.

Honors and Awards: None

Publications: None

Serial No. NCI-5002 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Genetic, Structural and Functional Characteristics of Human Immunoglobulins and Related Proteins

Previous Serial Number: Same

Principal Investigator: William D. Terry, M. D.

Other Investigators: None

Cooperating Units: Dr. David Davies, Laboratory of Molecular Biology, NIAMD; Dr. George Glenner, Laboratory of Experimental Pathology, NIAMD

Man Years:

Total:	2
Professional:	1
Others:	1

Project Description:

Objectives: The primary objectives of this project are to study the genetics and functional characteristics of human immunoglobulins and relate these characteristics to molecular structure. Finding previously unrecognized forms or categories of immunoglobulin molecules is an important part of this work. As different forms of immunoglobulin molecules are recognized, attempts are made to detect genetic factors in order that the genetic map of structural cistrons coding for immunoglobulin polypeptide chains can be more fully developed. In addition, aspects of the chemical heterogeneity of immune globulins are being studied to improve our understanding of the mechanisms generating the heterogeneity and to determine the possible biologic significance of this heterogeneity. The different forms of immunoglobulin molecules continue to be studied for correlations between molecular structure and biologic functions such as antibody activity, reaginic activity, etc. To further understanding of the functional significance of the classes and subclasses of immunoglobulins use will be made of "experiments of nature" leading to disease states characterized by selective immunoglobulin deficiencies. Patients with these diseases

will be studied for functional aberrations of their immune systems. In addition, the structure of amyloid, a protein deposited in the tissue of some patients with the above diseases, will be determined.

Methods Employed: Studies required isolation of immunoglobulins from normal serum and also from the serum and urine of patients with multiple myeloma and macroglobulinemia. Isolation and analytic techniques included ion exchange chromatography, gel filtration, preparative electrophoresis, immune precipitation in gel, autoradiography and analytic ultracentrifugation. Protein structural studies utilized paper chromatography, high voltage electrophoresis, the amino acid analyzer and the automatic amino acid sequencer. Ultracentrifugation, including both sedimentation and equilibrium techniques, has been used to characterize several systems.

Major Findings: Three-dimensional structural studies of the crystalline human IgG protein have not progressed beyond the construction of a 6Å model. Inherent disorder in this crystal limits the possible resolution and although attempts are being made to obtain other substitutions, it appears unlikely that additional significant progress will be made. These studies are, therefore, being terminated.

Amino acid sequence studies of specifically purified rabbit antibodies against haptenes have been carried out with Dr. Murray Freedman. Results indicate that conventionally prepared antisera contain limited sequence heterogeneity in the amino-terminal portions of their light polypeptide chains.

Structural studies of the IgE heavy chain have been terminated.

Structural studies of amyloid proteins have revealed two classes of amyloid. One is related to immunoglobulins and appears to contain large portions of, or entire, light polypeptide chains. The other class consists of a protein which has been sequenced, but which does not appear to be homologous with known immunoglobulins.

Significance to Biomedical Research and the Program of the Institute: The development of a more complete understanding of the operation and control of the immune system requires that the component parts of the system be identified as precisely as possible. In the past, this work has depended heavily upon the utilization of myeloma protein which can be isolated in a more homogeneous state than their normal counterparts in normal human serum. The finding that certain myeloma-like proteins both have antibody activity and form crystals under certain circumstances, leads to the exciting possibility of obtaining crystalline antibody molecules and of being able, therefore, to determine the three-dimensional structure of antibody molecules. It is only when both three-dimensional and primary sequence information can be put together that it will be possible to fully understand the nature of the interaction between antibodies and their antigens.

The generation of molecular variability among antibody molecules is not understood. Many theoretical formulations have been proposed and some of these can be subjected to critical testing. The accumulation of information concerning the primary amino acid sequences of the variable regions of heavy chains from the different classes and subclasses of immunoglobulin molecules should establish further constraints on the types of theories that may legitimately be entertained.

This project supports Objective 5, Approach 4.

Proposed Course: Attempts to identify crystallizable myeloma proteins with antibody activity will be continued. Additional studies on the structure and pathogenesis of amyloidosis will be pursued.

Honors and Awards: None

Publications:

Morell, A., Skvaril, F., Steinberg, A. G., van Loghem, E., and Terry, W. D.: Correlations between the concentrations of the four subclasses of IgG and Gm allotypes in normal human sera. J. Immunol. 108: 195-206, 1972.

Davies, D. R., Sarma, V. R., Labaw, L. W., Silverton, E. W., and Terry, W. D.: Three-dimensional structure of immunoglobulins. In Amos, B. (Ed.): Progress in Immunology. New York, Academic Press, 1971, pp. 25-32.

Sarma, V. R., Davies, D. R., Labaw, L. W., Silverton, E. W., and Terry, W. D.: Crystal structure of an immunoglobulin molecule by X-ray diffraction and electron microscopy. Cold Spring Harbor Symp., in press.

Glenner, G. G., Ein, D., Eanes, E. D., Bladen, H. A., Terry, W. D., and Page, D. L.: Creation of "amyloid" fibrils from Bence Jones proteins in vitro. Science 174: 712-714, 1971.

Polmar, S. H., Waldmann, T. A., and Terry, W. D.: Immunoglobulin E in immunologic deficiency disease: I. Relation of IgE and IgA to respiratory tract disease in isolated IgE deficiency, IgA deficiency and ataxia telangiectasia. J. Clin. Invest. 51: 326-330, 1972.

Glenner, G. G., Ein, D., and Terry, W. D.: The immunoglobulin origin of amyloid. Amer. J. Med. 52: 141-147, 1972.

Serial No. NCI-5003 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cellular Immunity I

Previous Serial Number: Same

Principal Investigator: John R. Wunderlich, M.D.

Other Investigators: John Martin, Ph.D., John Macdonald, M.D.

Contractor: None

Cooperating Units: None

Man Years:

Total:	5
Professional:	3
Other:	2

Project Description:

Objectives: The project has been directed at understanding and manipulating mechanisms of cell-mediated immune cytotoxicity. Long range goals are: 1) to identify and characterize factors which influence induction, maturation, and expression of a cellular immune response; 2) to define changes in cellular immunity which occur in tumor-bearing hosts; and 3) to find means of manipulating the cellular immune response for therapeutic purposes. Immediate objectives have been 1) to further characterize cytotoxic lymphoid cells, 2) to identify mechanisms in tumor-bearing hosts which may impede development of cytotoxic lymphoid cells, and 3) to characterize possible pathways for increasing the development of cytotoxic lymphoid cells in tumor-bearing hosts.

Methods Employed: Direct cell-mediated cytotoxicity has been measured in vitro with the assumption that activity reflects cytotoxic cellular immunity in vivo. Cell-mediated cytotoxicity is measured by the release of ^{51}Cr from labeled target cells incubated with immune lymphoid cells. Immune cells are collected from spleen, marrow, lymph nodes, thymus, and peripheral blood. Target cells are provided by tumors, tissue culture lines, and lymphoid cells from peripheral blood or nodes. Cytotoxic immune cell activity is enriched by BSA density gradient separation of cells and adoptive transfer of sensitized cells into X-irradiated syngeneic hosts. Lymphoid cells have also been sensitized in vitro using Mishell and Dutton tissue culture techniques.

Major Findings:

Mechanisms of Target Cell Destruction by Direct Cell-Mediated Cytotoxicity: Last year we found that direct cell-mediated cytotoxicity could be increased several hundred-fold by an adoptive transfer procedure. BALB/c mouse spleen cells sensitized against an allogeneic C57BL/6 lymphoid tumor, EL-4, were transfered with fresh tumor into X-irradiated syngeneic hosts. Spleen cells taken from the hosts 6 days after the transfer procedure were highly enriched for direct cell-mediated cytotoxicity and thus provided a "purified" source of immune cells for further study. Several findings have emerged from experiments with these immune cells: 1) purified immune cells mixed in vitro with an excess of chromium-labeled tumor cells destroy more tumor cells than the total number of spleen cells present. The average viable nucleated spleen cell destroyed up to 1.5 tumor cells in 4 hours. An unrelated tumor derived from BALB/c mice, LSTRA, was not damaged by the immune cells; 2) no evidence has been found for cell-free mediators of cytotoxicity released during 4-hour incubation periods: lymphotoxin, lymphocyte-dependent antibody, or complement-dependent antibody. Thus, in vitro tumor cell destruction in the system appears to depend upon direct cell-mediated cytotoxicity alone: 3) most important, incubation of ^{51}Cr-labeled immune cells with unlabeled EL-4 tumor cells has failed to show detectable immune cell damage during the cytotoxic reaction.

The apparent stability of immune cells during tumor cell destruction has been further investigated under in vitro conditions simulating the internal milieu of a tumor mass. BALB/c anti-EL-4 immune cells are not inhibited by cell-free extracts of necrotic EL-4 nodules or by dead EL-4 cells purified by discontinuous BSA density gradient separation. Relative to physiologic conditions, immune cells lose less than 5% activity in cytotoxic reactions at pH 7 or at 41°C. Interpretations of these results must be guarded because of the limited incubation time for cytotoxic reactions (2-4 hours), and the fact that experiments have been limited to one tumor system. However, it is encouraging that cytotoxic immune cells appear stable during target cell destruction and that the development of immune cells can be greatly accentuated by removing inhibitory mechanisms existing in natural hosts.

Suppressed Development of Cytotoxic Lymphoid Cells in Tumor Immunized Mice: The host environment which inhibits development of cytotoxic immune cells has been manipulated in C57BL/6 mice immunized with X-irradiated syngeneic EL-4 tumor cells. Mice immunized in vivo develop neither cytotoxic antibody nor, apart from an occasional small transient response, do they develop specific cytotoxic immune cells. Last year we were able to amplify the cellular immune response in C57BL/6 mice by chemically coating X-irradiated El-4 tumor cells with Concanavalin A (Con A). Mice sensitized and challenged with X-irradiated Con A-coated tumor developed significant cellular immune activity against native EL-4 tumor. Several new findings have advanced our understanding of this observation : 1) Con A does not serve as a carrier molecule for tumor-associated antigen. Presensitization of mice with Con A does not increase responsiveness to X-irradiated Con A-coated tumor. Also,

sensitization of mice with X-irradiated, Con A-coated tumor, followed 3 weeks later by challenge with X-irradiated tumor without Con A, evokes a cellular immune response greater than that evoked by inclusion of Con A on both the sensitization and challenged preparations of tumor. 2) Con A acts nonspecifically on the immune system, rather than solely on tumor cell antigens. Effective sensitization to EL-4 occurred when X-irradiated uncoated EL-4 cells were given with Con A-coated onto a human tissue culture line, IM-9. 3) "Riding in" of Con A on EL-4 or another unrelated cell type to appropriate areas of the host lymphon appears essential for sensitization. Thus, Con A administration alone, apart from EL-4, was not effective in evoking a cellular immune response to EL-4.

Stimulation of cellular immune responsiveness by coating tumor cells with Con A appears to occur because host suppressive mechanisms are overcome or bypassed. This conclusion stems from in vitro experiments with spleen cells from C57BL/6 mice. Several important observations have been made: 1) freshly explanted spleen cells from C57BL/6 mice sensitized with uncoated EL-4 were not cytotoxic for native EL-4 tumor. 2) normal C57BL/6 spleen cells responded to uncoated X-irradiated EL-4 tumor after several days of coincubation with EL-4 cells in tissue culture. 3) spleen cells from sensitized mice responded to EL-4 in vitro better than normal spleen cells. On some occasions additional EL-4 was not necessary to stimulate in vitro development of cytotoxic immune cells by sensitized spleen cells. Presumably, adequate antigen was carried over with presensitized spleen cells into the tissue culture system. In vitro stimulation of cellular immunity was specific. Cytotoxic immune cells did not damage an unrelated target cell (LSTRA) nor could unrelated tumor cells illicit immune cell activity against EL-4 target cells. These results show that C57BL/6 lymphoid cells can respond to EL-4 tumor and develop cytotoxic immune cells in the absence of host regulatory mechanisms. In vivo, an immune response is initiated as shown by a high capacity of sensitized spleen cells to produce cytotoxic immune cells in vitro. But maturation of the response is blocked in vivo.

Anomalous Cellular Immune Responses to Dead Tumor Cell Antigens: We investigated the possibility that host suppression of cellular immunity against tumors is related to tumor cell viability. The test system involved C57BL/6 derived EL-4 tumor cells which were killed by UV irradiation. Dead tumor cells were purified by a discontinuous BSA gradient. Dead tumor cells were unable to adsorb allogeneic complement-dependent antibody or react with and inhibit cytotoxic lymphoid cells. Dead tumor cells were unable to evoke complement-dependent cytotoxic antibody or cytotoxic lymphoid cells in BALB/c hosts. Cell-free antigens containing H-2 determinants, however, were found in tumor-free mediatfollowing UV irradiation. Immunization of BALB/c hosts with this antigenic material evoked humoral but no cellular immunity against native EL-4 tumor cells. BALB/c hosts sensitized by cell-free antigens died from tumor growth when challenged with $5x10^7$ viable EL-4. By contrast, normal mice and mice preimmunized with viable tumor survived challenge with greater than 10^8 live tumor cells and demonstrated excellent cellular immunity. At this point our working hypothesis was that dead tumor cells are immunologically inert, but the dying cells released (shed) antigens which induce an immune

response inappropriate for tumor rejection; namely immunity in the absence of cellular immunity.

Additional studies have demonstrated that cell-free antigens from dead tumor cells initiate a cellular immune response in hosts previously unimmunized, but maturation of the response is inhibited. Thus, spleen cells from antigen-sensitized donors mixed in vitro with viable tumor cells produced in the course of several days cytotoxic cell activity far greater than that found with normal spleen cells. Permissive in vitro environments relatively free of inhibitory host mechanisms appear to permit differentiation of presensitized "memory" cells into effector cells.

Finally, host immunologic status affects the action of cell-free antigen. If the host has been presensitized with viable EL-4 tumor, challenge with cell-free antigens stimulates both humoral antibody activity and cytotoxic lymphoid cell activity. The basis for immunogenicity of cell-free antigen remains to be worked out.

Significance to Biomedical Research and Programs of the Institute: A growing body of evidence demonstrates that tumors are immunogenic, but that the host immune response is out of balance and inappropriate for tumor rejection. Suppressive host mechanisms prevent full development of the host cellular immune response, which at maturity would most likely provide a prime mechanism for tumor rejection. One arm of cellular immunity, namely, cytotoxic lymphoid cells, is being characterized in terms of its potential for destroying tumor cells. More important, with existing methods for immunizing cells in vitro, mechanisms for suppression of cellular immune responses are being identified and techniques for eliminating or bypassing suppression are being developed. This work supports Objective 6, Approach 4.

Proposed Course of Project: Work will be continued along the following lines of investigation: further understanding of 1) mechanisms of target cell destruction by direct cell-mediated cytotoxicity, 2) requirements for development of cytotoxic immune cells, 3) mechanisms for suppression of the cellular immune response, and 4) methods for bypassing or eliminating host suppression of cellular immunity. A special effort will be made to correlate in vitro and in vivo activity of cytotoxic lymphoid cells.

Honors and Awards: None

Publications:

Wunderlich, J.R., Rogentine, G.N., Jr., Yankee, R.A.: Rapid in vitro detection of cellular immunity in man against freshly explanted allogeneic cells Transplantation 13:31, 1972.

Serial No. NCI-5004 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Regulation of Growth and Biosynthetic Activity of Cells of the Immune System

Previous Serial Number: Same

Principal Investigator: Donald N. Buell, M. D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Others:	1

Project Description:

Objectives: 1) The development of large scale, long term cultures of functionally capable immune cells. 2) Understanding the mechanisms which regulate gene expression and the biosynthetic processes involved in the formation of antibodies and other immunoglobulins. 3) Delineate the factors which play a role in control of growth and proliferation of cells of the immune system.

Methods Employed: Tissue culture of human lymphoid cells is an important part of this work. Immunofluorescent cytology and radioimmuno-electrophoresis are used to identify the specific immunoglobulins produced. Rates of production and turnover are studied by continuous and pulse label incubations with radioactive amino acids, with analysis of incubation medium and cytoplasm using solid phase radioimmunoassay. SDS-acrylamide gel electrophoresis permits analysis of the synthesis and assembly of the subunit immunoglobulins chains. Standard counting techniques are used to follow cell growth. Incorporation of specific labeled precursors followed by autoradiography and scintillation counting are used to study rates of RNA and DNA synthesis, and to determine cell cycle parameters. Synchrony of cell growth is achieved by double thymidine and thymidine-colcemid blockade. Rate zonal centrifugation is used to separate cells according to size, cell cycle position and functional properties. The functional

status of ribosomes is assessed after disruption of cells with detergent and cytoplasmic separation on ultracentrifugal density gradients. Freeze-thaw elution and detergent solubilization permit isolation of plasma membranes.

Antibody secretion by individual cells of the functional murine myeloma MOPC-315 is measured by incorporating TNP coated sheep RBC's in a modified Jerne plaque assay. Hemagglutination with TNP-SRBC's, and direct binding of ^{3}H labeled TNP-ε-amino lysine, are used to quantitate antibody levels in serum and ascites fluid.

Major Findings: Techniques were developed which permit IgG synthesizing IM-9 cells to remain in a high density stationary phase with full viability for as long as 14 days. Under these conditions the cell cycle time was markedly increased and the G1 phase of the cycle was particularly prolonged to over 100 hours. Protein and immunoglobulin synthesis were reduced to near basal levels. These cells appear to be functionally equivalent to an in vivo population of resting lymphocytes which can be considered to be in a GO or markedly prolonged G1 state.

IM-9 cells were found to undergo a characteristic and reproducible transition from stationary phase to exponential growth during which they mimic changes seen in the activation of resting lymphocytes. Changes in DNA and RNA synthesis and alterations of cell cycle parameters were measured during transitional growth. Synchronous pulses of immunoglobulin production were seen, reminiscent of the synchronous events seen in the generation of antibody forming cells in early immune responses.

After cutback from stationary phase into fresh medium, IM-9 cells undergo a lag of 22 hours followed by a synchronous entry into mitosis. Full immunoglobulin synthetic capacity is attained during the first postdivisional G1 period. Studies using actinomycin-D to inhibit new RNA transcription indicate that long-lived immunoglobulin messenger RNA can persist through mitosis and be used in this postmitotic immunoglobulin synthetic period.

A combined fluorescent antibody - autoradiograph technique has permitted more precise definition of the onset of immunoglobulin synthesis within the G1 period of exponentially growing IM-9 cells. Onset of IgG synthesis appears six hours following mitosis, approximately three to four hours before the start of S phase DNA synthesis.

A useful murine myeloma model system (MOPC-315) has been developed which will permit further investigations of the interrelationships of cellular proliferation and immunoglobulin gene expression during plasma cell differentiation. An increased efficiency of antibody production can be seen with a slowing of tumor growth. This appears to be associated with cells spending a greater part of their time in the functional, immunoglobulin synthesizing G1 portion of the cell cycle. Most recently,

several continuous cell lines of MOPC-315 have been established. These tissue culture lines remain tumorigenic and will permit parallel in vivo and in vitro manipulations.

Human lymphoid cell lines have been found to fall into two categories when tested for surface insulin receptors. One group carries receptors of low, medium and high affinity as described for fat cells and liver cells. A second group appear to have no insulin receptors.

Significance to Biomedical Research and the Program of the Institute: 1) Synchronization: An important development is the technique for inducing established tissue culture lymphocytes to enter a resting system. Large populations of these cells can then be induced to enter the cell cycle synchronously at a point in the G1 phase. This technique will prove useful to all workers interested in studying lymphocyte function.

2) Growth Control: A totally in vitro investigation of the repressor mechanisms involved in turning off lymphoid cell proliferation, and also mechanisms involved in lymphocyte activation, is now possible using tissue culture cell lines.

3) Myeloma: In vivo growth of a murine myeloma appears to follow a pattern nearly identical to other transplantable tumors. The alterations of immunoglobulin gene expression seen during growth of these malignant cells will provide important information towards understanding their counterparts, the normal antibody producing cells.

4) Immunoglobulin Production: Immunoglobulin production appears to be an integral part of the cell cycle of lymphocytes. Furthermore, immunoglobulin synthesis per cell increases as the number of cell divisions increase, suggesting that relatively stable factors that promote Ig formation persist from one generation to the next in rapidly dividing populations. Investigation of such factors is now feasible.

5) Key questions in immunology and cell biology in general center upon the nature of the alterations in molecular activity of a cell attendant upon binding of a specific activator molecule to its cell surface membrane. The finding that some but not all lymphocytes possess insulin receptors may open up new directions of investigation in these areas.

This project supports Objective 6, Approach 1.

Proposed Course of Project: Explore culture condition that will increase rate for successful establishment of human cells in permanent tissue culture. Factors such as CO_2, nutrients, feeder layers, etc., will be investigated.

Develop cell separation techniques in order to obtain specific functional cells for establishment in long term culture and detailed study.

Develop culture techniques for maintaining fully functional cells in high density cultures in order to establish high level in vitro antibody and immune cell production.

Investigate the specific molecular events involved in the onset of immunoglobulin synthesis in synchronized cultures.

Ribosomal function in lag and stationary phases of culture growth will be compared as part of the investigation of factors determining specific protein production. Using a cell free system, ribosomal activity will be analyzed during in vivo differentiation of MOPC-315.

The effect of insulin on membrane-bound ATPase activity will be compared in lymphocytes with and without insulin receptors.

Honors and Awards: None

Publications:

Levy, J. A., Buell, D. N., Creech, C., Hirshaut, Y., and Silverberg, H.: Further characterization of the Wi-L1 and Wi-L2 lymphoblastoid lines. J. Nat. Cancer Inst. 46: 647, 1971.

Serial No. NCI-5006 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: 1) Transplantation Antigens
2) Acute Leukemia Associated Antigens
3) Characterization of Lymphocyte Dependent Antibody

Previous Serial Number: Same

Principal Investigator: Dean L. Mann, M.D.

Other Investigators: Sidney Stein, M.D., Israel Yust, M.D.
G. Nicholas Rogentine, Jr., M.D.,
Christopher B. Merritt, M.D.

Visiting Scientist: Annick Le Pourhiet, M.D.

Cooperating Units: Brigid G. Leventhal, M.D., Medicine Branch, NCI
Roger Halterman, M.D., Medicine Branch, NCI

Man Years:

Total:	5 1/2
Professional:	3 1/2
Others:	2

Project Description:

Objectives: The objectives of the studies are threefold: 1. a) to purify human and monkey transplantation antigens; b) to chemically, genetically and immunologically characterize these antigens; c) use soluble HL-A alloantigens to raise allotypic antisera in heterologous species; d) to define and characterize cell surface components that function as vital receptor sites and relate them to HL-A alloantigens. This work supports Objective 3, Approach 5. 2. a) Identification of an antigen associated with acute leukemia; b) isolation and characterization of this antigen; c) use of antigen to produce heterologous antiserums; d) identify factor(s) in serum of leukemic patients that inhibit and/or promote cell-mediated cytolysis. This work supports Objective 4, Approach 1. 3. a) identification and characterization of a serum component (antibody) mediating cellular cytotoxicity (LDA); b) to screen sera from patients with autoimmune disease and malignancies for the presence of this antibody. This work supports Objective 6, Approach 4.

Methods Employed: ^{51}Cr release and dye exclusion cytotoxicity (See Project Report NCI-5008) were used to detect alloantigens. Human cells were obtained from normal blood bank donors and from tissue culture. Tumor cells, lymph nodes and spleen cells were used for assaying mouse H-2 alloantigens. Antisera detecting genetically determined human leukocyte alloantigens were obtained as described in Project NCI-5008. Mouse alloantisera were prepared in the conventional way. Alloantigens in subcellular form were detected by a cytotoxic inhibition assay using the ^{51}Cr release from target cells and employing the human leukocyte typing antiserums.

Soluble human leukocyte alloantigens were prepared by graded hypotonic lysis of cell suspensions, recovery of lipoprotein membrane fragments and papain digestion of these fragments to yield a water soluble antigen. Further purification was done by CM-Sephadex, gel filtration chromatography electrofocusing and acrylamide gel electrophoresis. Monkey transplantation antigens were solubilized and purified by the same method. A number of different techniques were employed to solubilize HL-A alloantigens. These included detergents, choatropic agents 3M KCl and sonication. The physical-chemical properties of the alloantigens were investigated.

To aid in studies of identification and isolation of HL-A alloantigens, heterologous antiserums were raised by injecting purified HL-A alloantigens into rabbits. These antiserums were evaluated for cytotoxicity to human lymphoid cells as well as for their ability to precipitate solubilized HL-A alloantigens. The antisera was absorbed with cells in attempts to raise specific allotypic antisera. (Collaborative studies Dr. F. Carey, St. Louis University School of Medicine.) The heterologous antiserums to cell membrane components were used in studies to attempt to block specific viral attachment to Hela cell

The acute leukemia associated antigen(s) was detected in dye exclusion and ^{51}Cr chromium release cytotoxicity assays. Antiserum used in these studies were obtained by immunizing rabbits with water soluble cell membrane preparations from the tissue culture cell line RAJI. Cell mediated cytotoxicit studies were performed by the method described by Wunderlich. Peripheral blood lymphocytes from patients with acute leukemia were labeled with ^{51}Cr, incubated with autologous serums or serum from other leukemic patients and/or with the heterologous antiserum that detect the acute leukemia antigen. Normal peripheral blood lymphocytes were added and the lysis or inhibition of lysis mediated by the various human serums determined.

Serums were obtained from patients who had received multiple transfusions or had a diagnosis of autoimmune disease or malignancies. These serums were tested in the cell-mediated cellular cytotoxicity reaction using various target cells and normal peripheral blood lymphocytes. The serums tested from the various patients were screened for their ability to mediate the cellular cytotoxicity reaction (LDA). The positive sera were fractionated by gel filtration, ion exchange chromatography, and electrofocusing. These fractions were tested for LDA to determine the immunoglobulin component responsible for this antibody activity.

Major Findings: New methods were explored to prepare soluble cell membrane antigens (HL-A) from tissue culture cell sources as well as from peripheral blood lymphocytes, particularly from patients with acute leukemia. Concentrated efforts were made to solubilize these antigens using non-ionic detergents. Sixteen different non-ionic detergent preparations were used and it was found that the detergent NP-40 gave highest specific activity for HL-A alloantigenic materials. Attempts were made to fractionate this material to ascertain the molecular characteristics of the cell membrane antigens solubilized by these methods. Immune precipitation tests were initiated and it has been observed that the heterologous antisera raised against soluble cell membrane preparations precipitated the active antigenic component from these soluble preparations. This is a major advance in this area, indicating that specific immune complexing can be used to isolate the HL-A antigenic materials.

Antiserums obtained from rabbits immunized with the soluble cell membrane component from the cell line RAJI were found to detect an antigen which is associated only with acute leukemia cells. Peripheral white blood cells from patients in remission and from normal peripheral blood lymphocytes could not be demonstrated to contain this antigen. Cultured cell lines derived from various neoplasms were also tested for the presence of this antigen and found to be negative. This antigen could be demonstrated to be induced in human embryonic kidney cell lines coincident with infection with murine Rauscher leukemia virus.

In the studies investigating LDA and blocking factors in serums from acute leukemia patients, specific immune reactivity was demonstrated in approximately one-third of the patients. However, in the majority of the patients tested thus far it appears that the serum contains a component which inhibits the reactivity of the LDA activity of heterologous antisera. Other investigators have demonstrated blocking factors in neoplastic disease which may explain the lack of immune reactivity of patients against their developing neoplasms. It appears that there is a blocking factor also in patients with acute leukemia.

In studies attempting to isolate and characterize LDA it has been demonstrated that patients receiving multiple transfusions have highest titers of this serum component. These sera were fractionated by gel filtration and ion exchange chromatography as well as electrofocusing. When these fractions were tested it was found that the maximum LDA activity did not correspond with maximum protein concentrations of IgM, IgA, IgG. These findings suggest that the LDA may be a subclass of IgG, or indeed, may be a new class of immunoglobulins.

Significance to Biomedical Research and the Program of the Institute: The preparation and analysis of soluble human transplantation antigens has both fundamental and clinical importance. Information gained as to the character of these antigens and the nature of the integration of these materials into the cell membranes structures will add to our knowledge of genetic

control as well as fundamental knowledge in basic cell membrane structure. In a clinical situation these soluble transplantation antigens could be used to induce tolerance or enhancement of allografted tissue and thus obviate the need for hazardous immunosuppressive agents.

The demonstration that acute leukemia has associated specific antigen or antigens is fundamental to understanding etiology and possible therapeutic approaches to treatment of this disease. The appearance of neo-antigens on cell surface has been well demonstrated in a variety of tumor systems in lower animals.

The demonstration that acute leukemia cells possess what appears to be specific antigenic determinants may allow a search for an etiologic agent which will cause this antigen to appear on acute leukemia cells. Immuno-therapy has been proposed in the acute leukemias. Such a program implies that acute leukemia cells do possess a particular antigen, which is shared by the cells from a number of patients with this disease. Our demonstration that there is indeed an acute leukemia associated antigen makes more rational the treatment of this disease by these means. The demonstration that there are substances in the acute leukemia serum which either block LDA activity or mediate such activity suggests a fine balance between immunity and "tolerance" in the patients with this disease.

The isolation and characterization of the lymphocyte dependent antibody is a relatively new area for exploration. The LDA appears to be an antibody which is not correlated with complement dependent cytotoxic serum components and may thus represent a new mechanism for destruction of tissues by the host's own lymphocytes. Investigation of the nature of this antibody and its specificity of reaction should contribute to our knowledge of immune reactions in patients with autoimmune disease as well as those individuals who possess malignancies.

Proposed Course of Project: Further chemical and biologic characterization of soluble HL-A alloantigens will be undertaken. Studies will be designed to isolate the antigenic determinants by immune precipitation techniques and to characterize this isolated product. Studies will be continued testing solubilized histocompatibility antigen for possible use in clinical situations for the induction of tolerance and/or enhancement.

Studies will continue in the isolation and characterization of the antigen associated with acute leukemia cells. In addition, efforts are currently being made to screen a number of the known oncogenic RNA viruses to attempt to correlate infection with these viruses and the appearance of antigen on cell surfaces. The various factors in the serum from leukemic patients which are either blocking or enhancing cell mediated cytotoxicity reactions will be isolated and characterized.

Further studies will be undertaken in an attempt to identify the specific serum component responsible for the LDA activity.

Honors and Awards: None

Publications:

Einstein, A.B., Mann, D.L., Gordon, H.G., and Fahey, J.L.: The immune reactivity of heterologous antisera against solubilized lymphoid cell membrane component. Tissue Antigens 1: 209-218, 1971.

Einstein, A.B., Mann, D.L., Ficker, S., and Terry, W.D.: Antiserum to soluble lymphoid membrane preparation stimulates human leukocyte DNA synthesis. J. Immun.107: 1205-1208, 1971.

Einstein, A.B., Mann, D.L., Gordon, H.G., Trapani, R.H., and Fahey, J.L.: Heterologous antisera against specific HL-A alloantigens. Transplantation 12: 299-304, 1971.

Mann, D.L., and Fahey, J.L.: Transplantation Antigens. Ann. Rev. of Microbiol. 25: 679-710, 1971.

Rosenberg, E.B., Mann, D.L., Hill, J.J., and Fahey, J.L.: Prolonged allograft survival in mice pretreated with soluble transplantation antigens. Transplantation 12: 402-405, 1971.

Mann, D.L., Halterman, R., Rogentine, G.N., Jr., and Leventhal, B.G.: Detection of an acute leukemia associated antigen. Science 174: 1136-1137, 1971.

Whisnant, J., Mann, D.L., Rogentine, G.N., Jr., and Robbins, J.: Human cell surface structures related to haemophilus influenze type B disease. Lancet 2: 895-898, 1971.

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Transplantation of Hematopoietic Cells in Humans

Previous Serial Number: Same

Principal Investigator: G. Nicholas Rogentine, Jr., M. D.

Other Investigators: Christopher B. Merritt, M. D.

Cooperating Units: Edward Henderson, M. D., Brigid Leventhal, M. D., Ronald A. Yankee, M. D., Medicine Brance, NCI; Robert Graw, M. D., Human Tumor Biology Branch, NCI; R. J. Trapani, Ph. D., Microbiological Associates, Inc.

Man Years:

Total:	1.5
Professional:	.5
Others:	1.0

Project Description:

Objectives: The primary objective of this study is to study ways to obtain successful long term bone marrow grafts in patients suffering from aplastic anemia, immunological deficiency syndromes or cancer. A part of this study is to test whether selection of histocompatible donor-recipient pairs will ensure successful bone marrow engraftment. This entails the establishment of reliable means of histocompatibility testing. Another part is to study newer ways of immunosuppressing the recipient to obtain a graft and prevent graft versus host reaction (GVHR).

Methods Employed: Histocompatibility testing was carried out by studying genetically determined HL-A alloantigens on human lymphocytes. These antigens are assayed in vitro by the Terasaki microcytotoxicity test. Other tissue typing techniques such as dye exclusion and ^{51}Cr release cytotoxicity and quantitative complement fixation tests are available in the laboratory. All typing for transplantation is currently conducted under contract at Microbiological Associates, Inc., under the direction of Dr. R. J. Trapani.

HL-A antigens are detected by human alloantisera. We have antisera recognizing twenty HL-A antigens in our panel at the present time. The source of these antisera has been described in Project Report NCI-5008, 1967-1968.

Horse antihuman thymocyte sera was obtained (under an IND) from the Upjohn Company for use as an immunosuppressant. This preparation is currently being tested for its efficiency in suppressing rejection of kidney and marrow grafts at other transplant centers.

Major Findings: The Relationship of Histocompatibility Matching to Bone Marrow Engraftment: About 50 families and 2000 unrelated individuals were histocompatibility typed this year for possible bone marrow transplantation, or platelet or leukocyte transfusion. Several donor-recipient pairs were identified who were HL-A identical. This identity was confirmed by mixed leukocyte culture (done by Dr. Brigid Leventhal). Of this group of HL-A identical donors and recipients, two received bone marrow transplants after either combination chemotherapy (cytoxan, BCNU, AraC, 6 Thioquanine) (one case) or antilymphocyte globulin and X-ray (one case). One of these patients, a young man with aplastic anemia, was on the Immunology Branch service, and the other who had acute leukemia, was on the Leukemia Service, NCI. The patient with aplasia died on day 28 with pseudomonas pneumonia and septicemia. GVHR was not seen. This is the second patient on our service to be engrafted with the use of antilymphocyte serum as part or all of the immunosuppression. The patient with acute leukemia underwent severe late GVHR but recovered and is alive and free from leukemia four months after transplantation; he is the only surviving marrow transplant recipient at present. The occurrence of GVHR in an HL-A identical donor-recipient situation confirms our own prior findings (NCI-5008, 1968-1971) and those of others. At the present time we have no way of predicting which HL-A matched recipients will get GVHR.

These two cases demonstrate that successful engraftment can be achieved with the use of two newer modes of immunosuppression. Both were attended by severe toxicity, however, and further trials of other immunosuppressant regimens are needed.

The Bone Marrow Transplantation Group, consisting of senior investigators from the Immunology, Human Tumor Biology, and Medicine Branches of NCI, continues to meet weekly to discuss transplant cases and pertinent experimental results.

The histocompatibility testing laboratory at Microbiological Associates, Inc., under the direction of Dr. R. J. Trapani is typing an average of 16 individuals' lymphocytes for HL-A antigens per day. The level of work from the contractor has been good and we anticipate a satisfactory future course. This facility does all the typing for marrow transplantation and the typing of blood bank donors and others for platelet and white cell transfusion (see Project Report of Dr. Ronald A. Yankee, Medicine Branch, NCI, 1971).

Significance to Biomedical Research and the Program of the Institute: Transplantation of hematopoietic cells in man is urgently needed as a means of correcting marrow aplasia. It is of particular relevance to other programs in the National Cancer Institute, as marrow aplasia is often seen in patients who have received high doses of chemotherapeutic agents or radiation for malignancy. The selection of compatible donor-recipient pairs is of prime importance in assuring graft acceptance and minimizing graft versus host disease. The success of the clinical marrow grafting program to date can be attributed in large part to proper selection of donor-recipient pairs.

A possible other use of marrow transplantation in patients with malignancies is to turn the GVHR against the tumor (and away from the normal host tissues) thereby destroying it. Some recent experimental animal work has suggested the clinical feasibility of this immunotherapeutic approach.

One of the major problems in successful hematopoietic cell transplantation in man is the control of the graft versus host syndrome. While careful histocompatibility testing may prevent or reduce the severity of GVHR or convert it to a graft versus tumor reaction (GVTR), most patients needing marrow transplants will not have HL-A matched donors. Therefore, other investigations designed to prevent GVHR are needed to overcome this stumbling block before marrow transplantation can be widely used as a treatment for marrow aplasia. The development of animal model systems is an essential element for preclinical trials of newer methods of suppressing GVHR. Progress on these model systems is discussed in Project Report NCI-5011, 1972. When results from these animal model systems are available, they will be translated into clinical practice.

This project supports Objective 6, Approach 1.

Proposed Course of Project: Patients with aplastic anemia, disseminated malignancies and immune deficiency syndromes will be admitted to the Immunology Branch for transplantation of hematopoietic cells. Bone marrow, fetal thymus and peripheral blood leukocytes will serve as sources of transplantable cells. Donors and recipients will be typed for leukocyte and erythrocyte antigens and compatible pairs will be selected. Transplantation may be performed in the absence of complete histocompatibility when sufficient animal model system data is available to suggest that new approaches to securing graft and preventing GVH will be successful in man. Particular emphasis will be placed on the use of antilymphocyte serum as an immunosuppressant.

Honors and Awards: None

Publications:

Leventhal, B. G., Mann, D. L., and Rogentine, G. N., Jr.: Sensitization to water soluble HL-A antigens. Transplantation Proc. 3: 243-245, 1971.

Graw, R. G., Leventhal, B. G., Yankee, R. A., Rogentine, G. N., Jr., Whang-Peng, J., Hertzy, G. P., Halterman, R. H., and Henderson, E. S.: HL-A and MLC matched allogeneic bone marrow transplantation in patients with acute leukemia. Transplantation Proc. 3: 405-408, 1970.

Merritt, C. B., Mann, D. L., and Rogentine, G. N., Jr.: Cytotoxic antibody for epithelial cells in human graft versus host disease. Nature 232: 638, 1971.

Krueger, G., Berard, C. W., DeLellis, R. A., Graw, R. G., Yankee, R. A., Leventhal, B. G., Rogentine, G. N., Jr., Herzig, G. P., Halterman, R. H., and Henderson, E. S.: Graft versus host disease: Morphologic variation and differential diagnosis in 8 cases of HL-A matched bone marrow transplantation. Amer. J. Path. 63: 179-202, 1971.

Wunderlich, J. R., Rogentine, G. N., Jr., and Yankee, R. A.: Rapid in vitro detection of cellular immunity in man against freshly explanted allogeneic cells. Transplantation 13: 31-37, 1972.

Kirkpatrick, C. H., Rich, R., Graw, R. G., and Rogentine, G. N., Jr.: Treatment of chronic moniliasis with lymphocyte transfusions. Lancet, in press.

Kirkpatrick, C. H., Rich, R., Graw, R. G., Smith, T. K., Mickenberg, I. D., and Rogentine, G. N., Jr.: Treatment of chronic mucocutaneous moniliasis by immunologic reconstitution. Clin. Exp. Immunol. 9: 733-747, 1971.

Graw, R. G., Yankee, R. A., Leventhal, B. G., Rogentine, G. N., Jr., and 18 other authors: Bone marrow transplantation in acute leukemia employing cyclophosphamide. Exp. Hematology 22: 118-125, 1972.

Serial No. NCI-5011 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Basic Immunological Studies in Hematopoietic Cell Transplantation.

Previous Serial Number: None

Principal Investigator: G. Nicholas Rogentine, Jr., M.D.

Other Investigators: Christopher B. Merritt, M.D., John R. Neefe, M.D., Dean L. Mann, M.D.

Cooperating Units: C. C. Darrow, III, Bionetics Research Laboratories, Inc., G. Herzig, M.D., Medicine Branch, NCI.

Man Years:

Total:	4
Professional:	2
Other:	2

Project Description:

Objectives: The objectives of this study are to investigate the central problem of transplantation immunology - the recognition and response to major and minor histocompatibility antigens - and to develop means to modify these processes. These studies are being carried out in rhesus monkeys undergoing bone marrow transplants and in which the graft versus host reaction (GVHR) serves as an excellent model for investigation of all aspects of transplantation immunology.

An important part of these studies is the identification of the transplantation antigen system of the animal species used, the Rhesus monkey.

Methods Employed: Experiments done in the past 12 months have employed a variety of techniques and methods. Femoral bone marrow obtained by aspiration from anesthesized donors is administered intravenously to lethally X-irradiated recipients. Such allografted monkeys are then medically supported and observed for the remainder of their life with frequent measurement of a number of clinical, biochemical, and immunologic parameters. In some cases frozen autologous marrow has been given. Lymphocyte antigens of the Rhesus monkey are studied by standard trypan blue or eosin dye lymphocytotoxicity tests employing rhesus alloantisera obtained from planned immunizations with

skin grafts and leukocyte injections. The mixed leukocyte culture technique is being adapted for use with monkey leukocytes and will provide another method of assessing histocompatibility.

Rhesus spleens obtained at operation have been used to prepare soluble lymphocyte antigens. Spleen cell membranes are digested by papain and purified by column chromatography and gel electrophoresis. Soluble antigens prepared in this manner are assayed in vitro for their ability to inhibit antisera against rhesus lymphocytes, and in vivo for their ability to cause accelerated rejection of rhesus skin allografts.

The production of anti-rhesus lymphocyte sera has continued with the immunization of rabbits and a horse with purified rhesus thoracic duct lymphocytes. The ALS resulting from these immunizations has been tested for activity in vitro, and for immunosuppressive properties in vivo using rhesus skin graft and GVHR systems.

Major Findings: We have extended the observation reported in the previous Project Report (NCI-5011, 1970-71) that antilymphocyte serum significantly prolongs survival of allografted monkeys when given to the recipient as one dose 24 hours prior to transplantation. To date twelve monkeys have been evaluated. Monkeys receiving ALS prepared in horses against rhesus or human thymocytes had less severe and less rapidly progressing graft versus host reaction than untreated recipients. ALS prepared in rabbits against rhesus thoracic duct cells was also effective in preventing the acute GVHR and allowed survival times four times greater than controls. In the ALS treated monkeys a subacute or chronic wasting syndrome was seen which was quite distinct from the severe acute GVHR seen in unmodified recipients.

We have further modified GVHR by use of an autologous marrow rescue technique. This is the first successful achievement of this in primates. Monkeys were prepared for transplantation with 900 rads total body irradiation and 24 hours later were grafted with allogeneic bone marrow cells. Three days after transplantation early manifestations of GVHR were present in some recipients. Cyclophosphamide, 75 mgm per kg, was given at this time and 24 hours later autologous marrow previously collected and stored at -170°C in 10% dimethylsulfoxide was given. This treatment in five of five animals prevented progression of the GVHR which in untreated recipients was uniformly lethal within two weeks. Three of these five animals are long term (>3 months) survivors and have evidence of only recipient hematopoietic tissue. Two died of marrow aplasia after the autograft, presumably due to too low a dose of autologous marrow. These latter findings vividly demonstrate the need for more investigation before clinical application.

Six new pools of rabbit antirhesus thoracic duct lymphocyte sera were produced and tested in vitro for lymphocyte toxicity. Titers ranged from 1:800 to 1:102,400. Skin graft prolongation was seen only in ALS with cytotoxicity titers greater than 1:800. These reagents are being tested at present for their ability to ameliorate GVHR.

Two more rhesus lymphocyte alloantigen groups have been defined by lymphocytotoxicity tests. Typing of over 150 nonrelated monkeys and over 30 pedigreed family groups clearly indicates the arrangement of these antigen groups in two closely linked sets of multiple alleles, analagous to the HL-A antigens of man. Amino acid composition of the solubilized purified lymphocyte antigens is strikingly similar to that of soluble HL-A and H-2 antigens.

In September 1971 three members of this research project took part in an international subhuman primate histocompatibility testing workshop held in Rijswijk, The Netherlands. The results of typing 16 families and about 90 unrelated monkeys with our reagents confirmed our own hypotheses and agreed closely with results obtained by the Rijswijk group. As a result of this workshop, the rhesus lymphocyte alloantigen system has been officially designated RhL-A.

Significance to Biomedical Research and the Program of the Institute: Research on the recognition and reaction to major and minor histocompatibility antigens is of fundamental importance in transplantation immunology. Furthermore, it has vital clinical importance. The National Cancer Institute has made a strong commitment to a clinical program of marrow transplantation. The first stage of this program is a reality. However, only HL-A matched donors have been used. This greatly limits the application of this form of therapy, as well over 60% of all potential recipients do not have matched donors. Transplantation of mismatched marrow has almost invariably resulted either in no graft take or fatal GVHR and even in perfectly matched donor-recipient combinations fatal GVHR may occur, although at a lower frequency. Our recent findings with respect to the modification of GVHR with ALS provides a tool for study of the basic mechanism of GVHR and also may be applicable clinically, particularly in situations where it is necessary to transplant marrow between individuals not perfectly matched at HL-A. Project Report NCI-5008 this year details the use of ALS and X-ray in the preparation of one patient for transplantation. This human trial was based on successful monkey experimentation. An understanding of the pathophysiology of GVHR obtained in this system will also be of benefit in planning new methods of intervention in this problem as it occurs in man.

Marrow transplantation may have a role in cancer immunotherapy. If a graft versus host (and also graft versus tumor) reaction could be established and then interrupted, a powerful antitumor effect might be seen. Our successful interruption of GVHR by the autologous marrow rescue technique lays the foundation for such studies.

This project supports Objective 6, Approach 1.

Proposed Course of Project: With respect to basic questions in transplantation, we will attempt to understand the pathophysiology of GVHR, as well as the mechanism by which ALS acts to modify the reaction. This will involve a number of observations comparing clinical, histopathological, and immunological data from ALS treated and untreated animals. Other methods will be

used, either alone or in combination, in an effort to further control the GVHR reaction. These will include further studies with soluble lymphocyte antigens in attempts to produce tolerance, and the use of blocking antibody to modify the reaction. The possibility of using nonlethal forms of immunosuppression to prepare the recipient for transplant will also be considered (such as the use of ALS and sublethal doses of X-ray). Autologous rescue experiments will be conducted to define the limits of this means of interrupting GVHR.

Further studies of monkey leukocyte antigens will be conducted using lymphocytotoxicity. New antisera will be produced by cross immunizing antigen matched monkeys. Special emphasis will be placed upon typing of families. Mixed leukocyte cultures will also be carried out in these families. The relationship of leukocyte antigens to graft success will be studied.

Honors and Awards: None

Publications:

Rogentine, G. N., Jr., Vaa., L., Ellis, E., and Darrow, C. C. II: Rhesus lymphocyte alloantigens: I. Identification of a major alloantigen system. Transplantation 12: 267, 1971.

Rogentine, G. N., Jr., Merritt, C. B., Vaal, L. A., Ellis, E., and Darrow, C. C. II: Alloantigens of Rhesus monkey lymphocytes. II. Serologic, genetic and chemical characteristics. Transplantation Proc. 4: 21-24, 1972.

Balner, H., Barnes, A. D., D'Amaro, J., Darrow, C. C., Demant, P., Gabb, B. W., Piazza, A., Metzgar, R. S., Seigler, H. F., Rogentine, G. N., Jr., van Rood, J. J., Ward, F., and Zweerus, R. L.: Joint Report of First Histocompatibility Workshop on Primates. Transplantation Proc. 4: 141-147, 1972.

Merritt, C. B., Darrow, C. C. II, Vaal, L., and Rogentine, G. N., Jr.: Bone marrow transplantation in rhesus monkeys following irradiation: Modification of acute graft versus host disease with antilymphocyte serum. Transplantation, in press.

Serial No. NCI-5014 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Lymphocyte Stimulation: The Chemical Basis of Gene Activation in Cellular Immunity

Previous Serial Number: Same

Principal Investigator: Steven A. Rosenberg, M.D., Ph.D.

Other Investigators: Ronald Levy, M.D., Bilha Schechter, Ph.D., N. Franklin Adkinson, M.D., William D. Terry, M.D.

Cooperating Units: None

Man Years:

Total:	3
Professional:	2
Other:	1

Project Description:

Objectives: 1) To chemically define the molecules involved in the early stages of lymphocyte activation in order to gain an understanding of the nature of the response of the sensitized lymphocyte to antigen. 2) To utilize these findings to develop sensitive assays for cellular immunity in rodents and man.

Methods Employed: Guinea pigs are immunized with protein antigens and serve as the source of lymphocytes for many of these studies. Mouse spleen cells and human peripheral blood cells are also used. The stimulation of protein synthesis by mitogens, specific antigen, or in mixed leucocyte interactions is determined by measuring the incorporation of radioactive leucine into cellular protein. Identification of chemical changes in proteins after lymphocyte activation has been measured using a double-label technique in the presence and absence of antigen or phytohemagglutinin. Lymphocyte proteins are then fractionated using gel filtration chromatography and polyacrylamide gel electrophoresis.

Major Findings: A rapid microassay for detecting cellular immunity to soluble antigens in rodents has been developed based on the ability of antigens to stimulate the protein synthesis of sensitized lymphocytes. This assay can

detect 0.1 nanograms of soluble antigen using as few as 5 x 10^5 lymphocytes. It can be completed in 7 hours, requires no serum, and is highly reproducible. Positive responses in this test exhibit carrier specificity and are thought to reflect cellular immune responses as based on experiments which show that reactivity cannot be transferred by serum, animals having humoral but not cellular immunity do not respond, and the pattern of response after immunization is different from that of humoral immunity. This technique has been extended to allow the rapid evaluation of mixed leucocyte responses in less than 24 hours. Efforts to extend this test to humans are in progress.

Phytohemagglutinin activation of lymphocytes has been used as a model of lymphocyte activation by antigen. The use of sensitive double-label techniques has enabled us to detect changes in protein synthesis of lymphocytes within 15 minutes after contact with phytohemagglutinin.

A specific molecular aggregate has been identified in and isolated from activated lymphocytes. This aggregate represents one of the earliest chemical changes occurring in the activated lymphocyte. Similar studies of antigen stimulated lymphocytes are in progress.

Significance to Biomedical Research and the Program of the Institute: Cell-mediated immune responses play an important role in the host response to tumors and transplanted tissue. Few methods currently exist for assaying these cellular immune responses, and the assay we have described should be a valuable one for this purpose. The speed, convenience, and reproducibility of the assay are assets that should prove useful for clinical studies. Because it measures the very early events in the activation of the lymphocyte, this assay might detect phenomena not heretofore detectable by other means.

The mechanism of the activation of immune lymphocytes represents an important gap in knowledge of the immune system. The molecules we are isolating from activated lymphocytes in our double-label studies may play an important role in the process of gene activation in the lymphocyte. The stimulation of lymphocytes by nonspecific mitogens and by specific antigens is also an excellent model for the study of gene activation and control, and thus parallels can be drawn to the regulation of gene function in differentiation, malignant transformation, and other cellular phenomenon. By studying the early protein changes associated with lymphocyte activation, we hope to gain insight into the general phenomenon of gene activation in mammalian cells.

This project supports Objective 3, Approach 4.

Proposed Course of Project: We are currently trying to extend to humans the assay we have developed for measuring cellular immunity. This assay will be used to detect and isolate tumor and histocompatibility antigens as well as to evaluate the host response to these antigens.

Fractionation of proteins synthesized early in the course of lymphocyte activation by both PHA and by specific antigen will continue with an emphasis on the biological role of these proteins.

Honors and Awards: None

Publications:

1. Rosenberg, S. A. and Levy, R.: Synthesis of nuclear proteins by lymphocytes within minutes after contact with phytohemagglutinin. J. Immunol., in press.

2. Levy, R. and Rosenberg, S. A.: The early stimulation of protein synthesis in sensitized guinea pig lymph node cells by antigen. J. Immunol., in press.

3. Rosenberg, S. A. and Levy, R.: A rapid assay of cell mediated immunity to soluble antigens based on the stimulation of protein synthesis. J. Immunol., in press.

4. Rosenberg, S. A., Levy, R., Schechter, B., Ficker, S. and Terry, W. D.: A rapid microassay of cellular immunity in the guinea pig and mouse. Transplantation, in press.

Serial No. NCI-5015 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Role of Sialic Acid in the Immune Response to Tumors

Previous Serial Number: Same

Principal Investigator: Steven A. Rosenberg, M. D., Ph. D.

Other Investigators: G. Nicholas Rogentine, Jr., M. D.; Albert B. Einstein, M. D.

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Others:	1

Project Description:

Objectives: 1) To define the chemical characteristics of sialic acid on the surface of human lymphoid cells. 2) To study the role of surface sialic acid on the immune recognition of cell surface antigens.

Methods Employed: Sialic acid is measured after hydrolysis in mild acid or after digestion with neuraminidase. Human lymphoid cells used in studies on the role of sialic acid in the HL-A antigen system are grown in continuous suspension culture using standard techniques. Synchronous cultures are induced using the double thymidine block technique. HL-A and other surface antigens of lymphoid cells before and after neuraminidase treatment have been quantitated using standard cytotoxic techniques and serial dilution of known antisera.

Major Findings: The kinetics of neuraminidase action on cell surface sialic acids has been studied. Eighty percent of the total human lymphoid cell sialic acid is on the cell surface membrane and is removed after 90 minutes treatment with neuraminidase (0.02 units/ml) at 37°C at pH 6.4. Under these conditions cell viability and growth is unaffected.

With Dr. Albert Einstein, it has been determined that the biosynthesis of the cell surface proteins containing sialic acid occurs within a 2-4

hour period in the G1 phase of the cell cycle just prior to the onset of mitosis. There are at least three major groups of membrane sialoproteins separable by gel filtration chromatography.

With Dr. G. Nicholas Rogentine, Jr., we have demonstrated that removal of sialic acid from the surface of cultured and fresh human lymphoid cells results in the uncovery of an antigen reactive with antibodies in normal rabbit serum. These antibodies can be removed from rabbit serum without removing complement by absorbing the serum with neuraminidase-treated cells in the presence of EDTA. We have further identified an antigen on the surface of neuraminidase-treated fresh human peripheral lymphoid cells that is capable of binding autologous circulating antibodies. These antibodies have been identified as IgM molecules and can bind complement to result in the lysis of the treated cell. These antibodies are present in all normal people but are absent from the sera of patients with agammaglobulinemia.

Significance to Biomedical Research and the Program of the Institute: Sialic acid has been demonstrated to be an important molecule affecting the host response to tumors. A knowledge of the chemical nature and biosynthesis of sialic acid on the cell surface will be of value in furthering the understanding of this phenomenon. The finding that humans possess natural antibodies capable of lysing neuraminidase-treated cells may be of importance as a mechanism of immune surveillance for the elimination of transformed, damaged or aged cells. These cells have been demonstrated to have altered patterns of sialic acid on their surface.

This project supports Objective 3, Approach 5.

Proposed Course of Project: To chemically define the antigens on the cell surface uncovered by neuraminidase and to study their biological role in tumor rejection.

Honors and Awards: None

Publications:

Rosenberg, S. A. and Einstein, A. B., Jr.: Sialic acids on the plasma membrane of cultured human lymphoid cells: Chemical aspects and biosynthesis. J. Cell Biol., in press.

Rosenberg, S. A., Plocinik, B. A., and Rogentine, G. N., Jr.: "Unmasking" of human lymphoid cell heteroantigens by neuraminidase treatment. J. Nat. Cancer Inst., in press.

Serial No. NCI-5016 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cellular Immunity II

Previous Serial Number: Same

Principal Investigator: John R. Wunderlich, M. D.

Other Investigators: Dean L. Mann, M. D.

Contractor: Bionetics Research Laboratory

Contract NIH-70-2207
Money Allocated:

This progress report represents only a portion of work supported by contract NIH-70-2207. Money allocated refers specifically to support for this project.

Cooperating Units: Michael Blaese, M. D., Metabolism Branch, NCI; Richard A. Malmgren, M. D., Laboratory of Pathology, NCI; John Potter, M. D., Georgetown University

Man Years:

Total:	2.5
Professional:	1.0
Others:	1.5

Project Description:

Objectives: The project has been directed primarily at understanding cell-mediated immune cytotoxicity in humans. The long range goals are: 1) to identify human cells capable of destroying foreign cells by cytotoxic, cell-mediated immune reactions; 2) to characterize factors which influence development of the cellular immune effector cells; 3) to characterize the mechanism of foreign cell destruction by effector cells; and 4) to define changes occurring in the cellular immune cytotoxic responses which accompany immune deficiency diseases, malignancies, transplanted organs, auto-immune states, and iatrogenic immune suppression.

Methods Employed: Direct, cell-mediated immune cytotoxicity is measured by the release of chromium 51 from labeled target cells incubated with immune lymphoid cells. Details of the assay utilizing human cells have been described elsewhere (Wunderlich et al., Transplantation 13: 31, 1972). Target cells are prepared from peripheral blood, pathological fluid exudates, tissue culture lines, and biopsy specimens of lymph nodes and solid tumors. Solid tissue samples and cells adhering to tissue culture bottles are mechanically separated. Aggregated cells are allowed to settle. Red cells are then removed by ammonium chloride Tris lysis. The resulting cell suspension is labeled with ^{51}Cr. Normal and immune lymphoid-attacking cells are prepared primarily from human peripheral blood. Heparinized peripheral blood is passed through nylon columns to remove polymorphonuclear cells and monocytes and then settled in plasma gel to reduce the red blood cell content. Recent studies with multiply-transfused humans have shown that cytotoxicity in this assay is specific for direct cell-mediated reactions and independent of conventional complement, humoral antibody, and nonspecific toxins.

Target cell preparation has been modified in order to study cytotoxicity involving lymphocyte dependent antibody (LDA). After chromium labeling, target cells are washed by centrifugation and then incubated with heat-inactivated serum (or plasma) containing LDA for one half hour. Incubation mixtures include carrageenan, a C1 attachment inhibitor. Target cells are again washed and then mixed with nonsensitized attacking lymphocytes in the presence of carrageenan.

Major Findings: Human Lymphocyte Dependent Antibody.

Previous work. During the previous year LDA was identified in a patient with choriocarcinoma. LDA was characterized by its utilization of normal lymphocytes to destroy specific target cells in vitro. LDA alone, with complement, or with monocytes was not cytotoxic. LDA was a 7S, IgG immunoglobulin and was specific for cellular antigens from the patient's husband. Presumably LDA represented an immune response against the choriocarcinoma tumor. This system was of particular interest because it represented a second mode of human cell-mediated cytotoxicity which could be monitored in vitro. In contrast to cell-mediated cytotoxicity with presensitized lymphocytes, LDA utilized normal lymphocytes to destroy target cells. Similar to cell-mediated cytotoxicity with presensitized lymphocytes, however, LDA-associated cytotoxicity did not require additional complement and did not liberate detectable nonspecific cell-free toxins. Also, kinetics of target cell destruction were strikingly similar for toxicity mediated by presensitized lymphocytes and for toxicity mediated by LDA cooperating with normal lymphocytes. We postulated that mechanisms for target cell destruction might be similar for the two modes of cell-mediated cytotoxicity following lymphocyte target cell contact. Further understanding of LDA-mediated toxicity thus appeared particularly important.

New findings. To clarify the role of attacking lymphocytes in cytotoxicity mediated by LDA, we investigated whether lymphocyte activation is necessary for toxicity. Five normal donors were identified whose cells were destroyed by LDA in the presence of lymphocytes from normal, allogeneic donors. When these LDA-coated target cells were mixed with syngeneic rather than allogeneic lymphocytes, LDA-coated target cells were destroyed equally well by syngeneic and allogeneic lymphocytes in each of the five cases. It seemed unlikely that lymphocytes utilized by LDA could have been activated against autoantigens. LDA, however, could have utilized lymphocytes activated against exogenous antigens such as viruses or bacteria. This possibility was further investigated in cooperation with Dr. Michael Blaese by using lymphocytes from five patients with Wiskott-Aldrich syndrome. This disease state is characterized by marked cellular immune deficiencies, and lymphocytes from these patients are presumably not activated against any antigen. Lymphocytes from these patients, nevertheless, were utilized by LDA for target cell destruction as effectively as normal donor lymphocytes. We concluded that lymphocytes utilized by LDA do not require previous activation.

Possible activation of lymphocytes during the cytotoxicity reaction in vitro was investigated by inhibiting protein synthesis with puromycin or cycloheximide. LDA-coated target cells were mixed with syngeneic lymphocytes which had been preincubated with puromycin or cycloheximide. Protein synthesis was less than 2% of control levels. Inhibitors of protein synthesis were also included in the cytotoxicity reaction. Results showed that cytotoxicity in the presence of protein synthesis inhibitors occurred at normal levels, even though protein synthesis had been effectively blocked. We concluded that lymphocyte activation, e.g. synthesis of new ribosomes, toxic proteins, etc., is not necessary for target cell destruction. Provided with target cell recognition molecules, such as LDA, nonsensitized lymphocytes appear fully equipped to destroy target cells.

The nature of lymphocytes utilized by LDA for target cell destruction was further investigated in cooperation with Dr. Michael Blaese by using cells from immune deficient patients. Evidence from patients with intestinal lymphangectasia suggested that lymphocytes lost into the bowel are recirculating lymphocytes; yet peripheral blood lymphocytes from five of such patients were more cytotoxic than normal lymphocytes for LDA-coated target cells. These findings suggest that cells utilized by LDA are non-recirculating lymphocytes. The state of maturity required for lymphocytes utilized by LDA was investigated with hypogammaglobulinemia patients with diminished IgG, IgA, and IgM in various combinations. Lymphocytes from these patients were utilized for target cell destruction, although not as well as lymphocytes from normal donors. Additional studies demonstrated that lymphocytes from a patient with Bruton's agammaglobulinemia, and a patient with Di George syndrome were utilized by LDA about one half as well as normal cells. Presence of any activity was of particular interest in these cases because it suggests primitive lymphocytes which have not differentiated into mature B or T cells can cooperate with LDA in target cell destruction.

Since lymphocytes from immunodeficient donors cooperate with LDA for target cell destruction, studies were extended to anergic cancer patients identified by their failure to respond to sensitization with DNCB. Lymphocytes from 5 anergic cancer patients were tested, and results showed that LDA-coated target cells were destroyed equally well by lymphocytes from normal donors and these patients. Lymphocytes from anergic cancer patients thus appeared fully equipped to destroy target cells if provided with recognition molecules. Recognition molecules such as LDA might be provided by species other than man, since previous studies have demonstrated that human LDA utilizes xenogeneic lymphocytes for destruction of human target cells. Preliminary studies indeed show that LDA can be provided by immunization of species other than man. Sera from rabbits immunized with human cells, for example, contain LDA which utilizes human lymphocytes for destruction of human target cells.

Other areas of research work. Stability of cytotoxic lymphoid cells from both mice and humans is being evaluated in vitro in order to predict effects of storage on cytotoxic function. Effects of pH and temperature on cytotoxic function are also of interest because the internal milieu of tumors in situ may deviate from normal physiologic conditions. Preliminary findings show that both human and mouse effector cells in tissue culture lose activity within a week. Cytotoxic activity, however, can be recovered from immune mouse cells frozen in liquid nitrogen, suggesting that prolonged storage of effector cell activity can be achieved. Additional studies have shown that cytotoxic cells from sensitized mice function in vitro at elevated temperatures (41°C) and at mild acid pH (7.0), suggesting that these conditions will not block immune activity in situ.

Human cell-mediated cytotoxicity against autochthonous tumors is being evaluated in collaboration with Drs. John Potter and Richard Malmgren. Methods have been developed for preparing single cell suspensions of viable tumor and using these as target cells for peripheral blood lymphocytes in short term cytotoxicity tests. On the basis of 40 solid tumor studies, cell-mediated cytotoxicity against autochthonous tumor have been detected in only five cases, and these cases involved gastrointestinal or lymphoid cell tumors.

Selected potential transplant recipients at Baltimore City Hospital and NIH are being evaluated for preexisting cytotoxic immune cells against donor cells. Cellular immune activity presumably would have a deleterious effect on graft survival.

Significance to Biomedical Research: Understanding the mechanism(s) of target cell destruction by cellular immune cells is particularly important in fields of cancer, transplantation, and auto-immunity. At least one of these mechanisms has been clarified by studying the function of lymphocyte dependent antibody (LDA). LDA serves as a recognition site allowing specific contact between lymphocytes and target cells, which is followed by target cell death. Since the "attacking" lymphocyte is nonsensitized and LDA by itself is

nontoxic, nonimmune cells including tumor cells, organ transplant cells, etc., would appear to have autodestruct mechanisms. We feel more attention should be directed toward the role of target cells in cellular immune reactions and possible autodestruct mechanisms within target cells which are triggered by cytotoxic immune cells. The function of LDA should also be considered from a practical standpoint. Nonhuman LDA specific for human tumor antigens, for example, may be useful in treatment of anergic cancer patients.

Knowledge of storage potential for cytotoxic effector cells and their precursor cells is needed in order to provide standardized reagents both for therapeutic and laboratory uses. The finding that mouse effector cells can be preserved in liquid nitrogen is encouraging.

This project supports the National Cancer Plan Objective 6, Approach 4.

Proposed Course of Project: Further effort will be directed at understanding the role of target cells in cell-mediated cytotoxicity reactions. Thus, susceptibility of various human tumors to lysis by a standardized source of xenogeneic immune cells will be studied. Short-term cytotoxicity tests will be continued with human lymphocytes and autochthonous tumors with particular emphasis on amplifying activity of effector cells. In addition, attempts will be made to prepare lymphocyte dependent antibody specific for tumor antigens. Such a reagent would provide both a sensitive method for detecting tumor-associated antigens, and perhaps it would be useful therapeutically.

Peripheral blood lymphocytes from multiply-transfused patients and other donors sensitized by pregnancy or auto-immune disease will be used as likely sources of sensitized precursor cells for cytotoxic lymphocytes. Thus, factors affecting the formation of effector cells in vitro can be evaluated. Assessment of selected potential graft recipients will be continued to determine if cytotoxic immune cells influence survival of grafts.

Honors and Awards: None

Publications: None

Serial No. NCI-5017 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of the Biological Significance of Cell Surface Antigens with Emphasis on Histocompatibility Antigens

Previous Serial Number: Previously part of Project Report NCI-5008

Principal Investigator: G. Nicholas Rogentine, Jr., M. D.

Other Investigators: Lloyd K. Everson, M. D., Steven A. Rosenberg, M. D., Ph. D., Dean L. Mann, M. D.

Cooperating Units: John Whisnant, M. D., John Robbins, M. D., NICHD; Myron Falchuck, M. D., NIAMD; Warren Strober, M. D., Ronald Yankee, M. D., Richard Simon, Ph. D., John Gart, Ph. D., NCI; Paul Gerber, Ph. D., DBS; Robert Trapani, Ph. D., Microbiological Associates, Inc.

Man Years:

Total:	3.5
Professional:	2.0
Others:	1.5

Project Description:

Objectives: The overall orientation of this project is to study the natural biological significance of cell surface antigens especially those of the major histocompatibility antigen system. It is known that these antigens are important in allografting, however, the emphasis of this project is to study their significance apart from transplantation.

One objective is to find associations between certain HL-A antigens and human diseases. Another objective is to study qualitative and quantitative changes of these antigens in normal and neoplastic cells in vivo and in vitro.

Methods Employed: HL-A antigens on cell surfaces are assayed by standard ^{51}Cr release or dye exclusion cytotoxicity tests. Estimates of HL-A antigen content of different cell populations are achieved by quantitative absorption of HL-A antisera and retesting the absorbed antisera against a standard target cell. HL-A typing of the lymphocytes

of patients with a variety of diseases is being conducted under contract at Microbiological Associates, Inc., under the direction of Dr. Robert J. Trapani.

Human lymphoid cells are maintained in long term tissue culture in the Branch. Techniques for obtaining synchronous populations of these cells are being developed so that the HL-A antigen content of a cell as a function of cell cycle phase may be studied.

Major Findings: HL-A antigens have been assayed in patients from several disease categories. These include acute lymphocytic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, chronic myelogenous leukemia, Hodgkin's disease, aplastic anemia, immunological deficiency syndromes, systemic lupus erythematosus, sprue, Hemophilus influenzae disease, pemphigus vulgaris and dermatitis herpetiforms. Significant associations of various HL-A antigens with certain diseases have been established: HL-A2 with acute lymphocytic leukemia, HL-A5 with Hodgkin's disease, HL-A8 with lupus and sprue. In the latter case, sprue, this finding is particularly striking; 21 of 24 patients had HL-A8, compared to the expected 5 of 24. This finding has direct relevance to the pathogenesis of the disease and experiments suggested by this finding are underway.

A physical method of obtaining unperturbed cell cycle phase synchronized human tissue culture lymphoblasts has been developed. This method will allow us to study variations in HL-A and other cell surface antigens during the cell cycle. Furthermore, it would enable a variety of studies to be done on purified cells in different phases of the cell cycle which have been impossible to date because of inadequacies in other methods of cell synchronization.

Significance to Biomedical Research and the Program of the Institute: Knowledge of cell surface structures is of central importance in understanding immune phenomena, normal growth and differentiation and malignant transformation. Cell surface antigens are easily identified markers for surface structures. The cell surface histocompatibility antigens are a product of an extremely polymorphic genetic system which must have important survival value for the individual quite irrespective of their role in provoking allograft rejection. Investigations into the nature of these important antigens in normal and malignant cells, in different organ systems, in different phases of a cell cycle, and in different stages of cellular maturation will give important information which may help us to understand the basic processes of growth and differentiation, malignant transformation and immunological recognition.

The study of the associations of certain diseases with certain HL-A antigens has a two-fold significance if such associations are found. It will allow the identification of those members in the population who are

at a higher risk and thereby allow early diagnosis or by modifying environmental factors possibly prevent the disease. The second significance lies in pointing a way to design experiments that probe the basic pathogenesis of the disease.

This project supports Objective 3, Approach 5.

Proposed Course of Project: A variety of patients with diseases, especially malignancies and disorders of immunity will be typed for HL-A antigens. Correlations will be sought and experiments designed to probe the pathogenesis of the disease will be attempted.

HL-A antigens and other cell surface structures will be studied on human lymphoblasts growing in long term tissue culture. They will be studied during different phases of the cell cycle and under differing environmental conditions. When possible, comparisons of HL-A antigen content on normal and neoplastic cells will be made.

Honors and Awards: None

Publications:

Whisnant, J. K., Rogentine, G. N., Jr., Mann, D. L., and Robbins, J. B.: Human cell-surface structures related to Haemophilas Influenzae Type B disease. Lancet ii: 895-897, 1971.

Mann, D. L., Rogentine, G. N., Jr., Halterman, R., and Leventhal, B. G.: Detection of an antigen associated with acute leukemia. Science 174: 1136-1137, 1971.

Rosenberg, S. A., Plocinik, B. A., and Rogentine, G. N., Jr.: "Unmasking" of human lymphoid cell heteroantigens by neuraminidase treatment. J. Nat. Cancer Inst., in press.

Serial No. NCI-5018 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Characterization of Cell Surface Molecules on Human Lymphoid and Tumor Cells

Previous Serial Number: None

Principal Investigator: Pierre A. Henkart, Ph. D.

Other Investigators: William D. Terry, M.D., Dean L. Mann, M.D.

Cooperating Units: Charles W. Todd, Ph. D., Duarte, California

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: 1) To characterize chemically several biologically important molecules of the lymphocyte cell surface, using cultured human lymphoid cells. These receptors include sugar groups which are receptors for the mitogens Concanavalin A and phytohemagglutinin, and the proteins which are histocompatibility antigens. 2) To characterize chemically the carcino-embryonic antigen of human colon cancer.

Methods Employed: Large scale suspension culture techniques of established human lymphoid cell lines have been established to provide adequate amounts of cells. Crude membrane preparations are extracted with ionic and nonionic detergents to solubilize the lymphoid surface receptors which are then purified by column chromatography. Human histocompatibility antigens are assayed by their inhibition of specific cytotoxic antisera and the receptors for mitogens are assayed as described below.

In the case of CEA from colon tumors, the antigen is extracted using perchloric acid and partially purified using gel filtration. Activity is measured quantitatively by radioimmunoassay or qualitatively by Ouchterlony gels.

Major Findings: Over 20 nonionic detergents were screened for their ability to solubilize active HL-A antigens, with Triton CF 54 and Nonidet P40 proving best. The extract was then immediately chromatographed on Agarose,

which afford separation of the antigenic activity from the detergent and over half of the detergent soluble protein.

Crude lymphoid cell membrane preparations were shown to have receptors for the mitogens PHA and Con A by an absorption assay. The mitogen was mixed with the membranes and incubated; this was followed by a high speed spin to pellet the membranes and the supernatant was tested for mitogenic activity by looking for an increase in the rate of protein synthesis when an aliquot of the supernatant was mixed with guinea pig lymph node cells. The absorption of Con A by membranes could be specifically inhibited by α-methyl glucose.

Amino acid and amino sugar analysis of CEA purified by gel filtration on Sepharose 4B and Sephadex G-200 showed a normal distribution of amino acids, with an absence of methionine. Protein accounted about 50% of the dry weight, and amino sugars accounted for about 25% of the dry weight. Although these CEA preparations were previously believed to be homogeneous, we have shown that they contain noncross-reacting material which reacts with the lectins Con A, PHA and wheat germ agglutinin in agar gels.

Significance to Biomedical Research and the Program of the Institute:

Basic general knowledge of cell surface molecules of higher organisms is still rudimentary. At present it is unclear how many different molecules are exposed to the exterior and how they mediate their many functions of recognizing external molecules and other cells. At present progress is being made using red blood cells but it is not clear how similar these cells are to other types. I have chosen to study lymphoid cells because they are probably more typical of other cells in the body and because they can be grown in large quantities in culture.

Basic knowledge of the chemistry of cell surface molecules is needed in order to manipulate them in a rational way to obtain clinically beneficial results. In the case of tumor antigens, perhaps including CEA immunity directed against these antigens must be stimulated. The availability of pure and chemically defined tumor antigens may allow patients to be immunized against their own tumors. The goal of chemical work on transplantation antigens is to devise a method of inducing tolerance to these antigens in transplant recipients. Here again, rational methods of attempting this will be greatly aided by knowledge of the chemistry of these antigens, as well as the availability of purified material. Objective 3, Approach 5.

Proposed Course of Project: In all cases further work is needed to purify the surface receptor molecules described. Both standard techniques such as ion exchange chromatography and specific methods such as immunoprecipitation and affinity chromatography will be attempted. The purified preparations will be subjected to physical studies and chemical analysis followed by further studies to define the active site of the receptor.

Honors and Awards: None

Publications: None

Serial No. NCI-5019 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structural and Immunochemical Analysis of Membrane Proteins of Normal and Malignant Cells

Previous Serial Number: None

Principal Investigator: William D. Terry, M. D.

Other Investigators: Pierre A. Henkart, Ph. D., Richard W. Smith, M. D.

Cooperating Units: Vincent Marchesi, M. D., NIAMD; Charles W. Todd, Ph. D., City of Hope Hospital, Duarte, California

Man Years:

Total:	2.5
Professional:	1.5
Others:	1.0

Project Description:

Objectives: Attempts will be made to isolate and characterize membrane proteins which (1) appear to be associated with the malignant state, and/or (2) appear to be important in acquiring an understanding of membrane structure. Attempts will also be made to develop reagents directed against differentiation antigens.

Methods Employed: A variety of procedures are used to solubilize membranes, followed by isolation of protein components by gel filtration, column chromatography, isoelectric focusing, and other forms of preparative electrophoresis. Isolated proteins are assessed for purity by acrylamide gel electrophoresis, and immunochemical techniques. Amino acid compositions of proteins and enzymatically prepared fragments are determined on the amino acid analyzer. Sequence analysis is carried out with manual and automatic sequencing procedures. Immunochemical studies utilize complement dependent cytolysis assays.

Major Findings: 1) Carcinoembryonic antigen has been chosen as the first tumor associated antigen to be subjected to structural analysis. Seven different CEA preparations have been found to have similar amino acid

compositions. Two of these have been partially sequenced in the automatic amino acid sequencer and have been shown to have identical and homogeneous amino acid sequences.

2) A red cell membrane glycoprotein has been partially sequenced. The overall pattern found was one in which one end of the molecule contained all of the carbohydrate residues and an amino acid sequence of unremarkable nature. Two-thirds of the way to the carboxy-terminus of this molecule, a hydrophobic stretch of about 30 residues was identified.

3) An antiserum has been prepared against human fetal thymocytes and absorbed with long term lymphoid cell lines which have immunoglobulin on their membranes and are therefore considered to be analogous to B cells. The absorbed antiserum has relative specificity for thymus cells, as opposed to "B" cells.

Significance to Biomedical Research and the Program of the Institute: Characterization of tumor associated antigens is of major importance in determining the relationship of these structures to normal cellular membrane components. Knowledge of the complete structure of the protein portion of the CEA molecule will permit possible modifications of this molecule in attempts to improve therapy and/or diagnosis of certain cancers.

The structural information derived from the red cell membrane glycoprotein has provided for the first time experimental evidence supporting the theoretical model for membrane structure that has been proposed on the basis of indirect evidence.

The availability of reagents capable of distinguishing various populations of human lymphoid cells will help in assessing the role of these populations in host responses to tumors.

This project supports Objective 3, Approach 5.

Proposed Course of Project: Studies of the structure of both these membrane proteins will be continued and an attempt will be made to obtain complete sequences for both proteins. Additional characterization of the antithymus cell antiserum will be undertaken.

Honors and Awards: None

Publications: None

Serial No. NCI-5020 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunoprophylaxis and Immunotherapy of Tumors in Experimental Animals

Previous Serial Number: None

Principal Investigator: William D. Terry, M. D.

Other Investigators: Steven A. Rosenberg, M. D., Ph. D.

Cooperating Units: David Lavrin, Ph. D., Contract NIH-70-2207 with Bionetics Research Laboratories, Inc.

Man Years:

Total:	2.5
Professional:	1.0
Others:	1.5

Project Description:

Objectives: This is the first of a series of experiments designed to study the effect on primary induced tumors of various manipulations of the immune system.

Methods Employed: Tumors are induced in mice by a variety of methods, inducing injection of methylcholanthrene. Tumors are harvested and frozen for subsequent use. Animals are immunized by conventional techniques.

Major Findings: In the first set of experiments, animals were injected with BCG at various intervals relative to the injection of a tumor inducing dose of methylcholanthrene. Animals were observed for tumor development and time of mortality. It was found that BCG had a slight but definitely beneficial effect in terms of prolonging survival in all experimental groups except those receiving BCG at about the time the tumor became palpable. In this latter group, there was a slight shortening of survival.

Significance to Biomedical Research and the Program of the Institute: BCG is being used in human immunotherapy and it is important in both prophylaxis and therapy models to determine the possible beneficial and deleterious effects of this nonspecific stimulator of the immune system.

This project supports Objective 6, Approach 4.

Proposed Course of Project: A second study has been set up designed to generate "deblocking" factor and a therapy model utilizing an antigenic MCA induced tumor has been established. Attempts will be made to see whether the preliminary beneficial effects noted in rats can be duplicated in mice with established tumors who are treated with "deblocking" serum.

Immunotherapy studies will be extended to humans in a collaborative project with the Surgery Branch, NCI.

Honors and Awards: None

Publications: None

ANNUAL REPORT OF THE METABOLISM BRANCH
SUMMARY OF SIGNIFICANT ACTIVITIES
NATIONAL CANCER INSTITUTE

July 1, 1971 through June 30, 1972

The research of the Metabolism Branch has been directed toward two major goals. The first is to define host factors that result in a high incidence of neoplasia. In this area, a broad range of immunological investigations have been carried out with special emphasis on the study of patients with immunological deficiency diseases where the immunological surveillance mechanisms that are important in elimination of neoplastic cells do not function normally. The second major goal is to determine the physiological and biochemical effects that a tumor produces on the metabolism of the host. Both patients with neoplastic disease as well as those with non-neoplastic disorders that facilitate the development of techniques for the study of hemostatic mechanisms and metabolic derangements of these control mechanisms are being investigated.

Studies of the Physiology and Pathophysiology of the Immune Response

The pathophysiology of the immune defects have been studied in patients with 3 major categories of immune deficiency and increased incidence of malignancy. In the first exemplified by ataxia telangiectasia there is a defect in the development of effectors of the immune system; and in the second exemplified by intestinal lymphangiectasia there is a short survival of effectors of the immune system; in the third, exemplified by the Wiskott-Aldrich syndrome there is a defect in antigen processing and recognition.

A broad study of immunological function in 25 patients with ataxia telangiectasia was continued. Ataxia telangiectasia is an inherited multi-system disorder characterized by cerebellar ataxia occulocutaneous telangiectasia increased frequency of respiratory infections and a 10% incidence of neoplasia. Patients with this syndrome menifested a complex array of abnormalities of the synthesis of the elements of both the humoral and cellular immune systems. The majority of the patients lacked both serum and secretory IgA and IgE. These findings were accompanied by a variable antibody response to a variety of antigens with some patients responding quite poorly. The response in the IgA immunoglobulin class, assessed by a modification of the C'1a fixation and transfer test was absent, even in those patients whose serum contained IgA. Finally, the patients showed a particular unresponsiveness to viral antigens. In the cellular immune systems, the patient with ataxia telangiectasia responded poorly to skin test antigens, had a defect in in vitro lymphocyte transformation to a variety of antigens, and had an absent or poorly developed thymus. One abnormality that has been considered as a possible fundamental cause of the immunological defects is the partial failure of thymic development and function. In order to assess the relationship of the thymus to IgA immunoglobulin synthesis the effect of thymectomy on IgA antibody production was studied in rabbits using the C'1a fixation and transfer test. It was established that there is a greatly dminished IgA response to a variety of soluble antigens with little change in the IgG or IgM antibody responses to these agents. More recently it has been shown that IgA responses are enhanced if antigen is given in

Freund's adjuvant. This suggests that IgA responses are more dependent on thymic dependent helper cells than are the responses in other immunoglobulin classes.

A second abnormality that has been considered as a possible basic factor in the defects in patients with ataxia telangiectasia is a defect in the interaction between entodermal and mesodermal elements that is required for normal tissue differentiation. In this regard the patients make primitive low molecular weight IgM, do not produce IgA and IgE and have immature thymus glands. We have recently defined a new piece of evidence supporting this hypothesis of inadequate tissue maturation. That is, all 22 patients with ataxia telangiectasia studied have markedly elevated levels of the fetal protein α-fetoprotein whereas no patients with other immunological deficiency syndrome nor siblings of patients with ataxia telangiectasia manifest this abnormality.

Studies of the immunological defect in 25 patients with intestinal lymphangiectasia have been continued. Intestinal lymphangiectasia is a disease with a primary generalized disorder of lymphatic channel development. There is loss of both serum protein and lymphocytes into the gastrointestinal tract secondary to an abnormality of intestinal lymphatics. As a result of this loss of proteins the patients have reduced levels of all classes of long surviving immunoglobulins. The reduction is most profound in IgG which is normally catabolized at a rate of 6%/day whereas IgE with a normal catabolic rate of 72%/day is only minimally effected by the bulk loss process. Despite this loss the patients are able to make antibody responses to antigenic challenge. Cellular immunity is more profoundly depressed in these patients. As a consequence of lymphocyte loss the patients are profoundly lymphocytopenic. We have recently shown that loss of lymphocytes into the gastrointestinal tract in patients with lymphangiectasia results in a qualitative change in the remaining lymphocyte population as well as a reduced number of peripheral lymphocytes. That is, there is a relative depletion of the population of lymphocytes necessary for cell mediated function. This conclusion is based on studies of the in vitro blastogenic transformation of lymphocytes from 12 patients with intestinal lymphangiectasia. The peripheral blood lymphocytes from patients with intestinal lymphangiectasia had impaired in vitro transformation to non-specific and specific mitogens. The most likely explanation for the qualitative lymphocyte defect in these patients lies in the fact that the peripheral lymphocyte pool in man is a functionally heterogenous population of cells including one population of short-lived cells that do not recirculate to the lymph and long-lived population predominately a thymic-dependent population that recirculates from the blood through the lymphatics ultimately returning to the blood via the thoracic duct. This latter population of cells the one most involved with cell mediated immunity is particularly prone to the effects of chronic gastrointestinal lymph loss because of its pathway of recirculation is to the lymphatics where the loss is occurring and because of the normally long life span of these cells.

As a consequence of their lymphocytopenia and relative depletion in recirculating thymic-dependent lymphocytes patients with intestinal lymphangiectasia are unable to make delayed type immune responses and cannot reject skin grafts of

unrelated donors. Three of the patients with intestinal lymphangiectasia have developed lymphoma or reticulum cell sarcoma.

The Wiskott-Aldrich syndrome is a sex-linked recessive disease characterized by thrombocytopenia, eczema and a high incidence of infections. Four of the 13 patients studied with this syndrome at the National Institutes of Health have developed reticuloendothelial neoplasms. The patients with this syndrome were shown to have markedly increased rates of immunoglobulin synthesis especially of the IgA and IgE classes. However, they were unable to make antibody response to many antigens. They were also unable to manifest delayed hypersensitivity responses in vivo and had impaired in vitro lymphocyte transformation to specific antigen despite the fact that they have a normal thymus and near normal levels of lymphocytes that transform normally in vitro to non-specific mitogens. The broad immunological defects in these patients with normal effector lymphocytes and immunoglobulins led to the hypothesis that these patients have a disorder of antigen processing or recognition, a defect in the afferent limb of immunity.

In order to study the pathways involved in the afferent limb of immunity an experimental model was developed using neonatal rats and mice that are immunologically deficient. In addition to the key role in this development played by central lymphoid organs, (i.e., thymus) a variety of other factors were also found to be important in the initiation of normal immune responses. The degree of maturation of the macrophage-afferent limb of immunity was demonstrated to be the limiting feature in the expression of immune reactivity to many antigens in the naonatal period. The degree of macrophage maturation could be enhanced by a variety of agents such as BCG, pertussis vaccine and endotoxin, and these agents were therefore effective in promoting effective immune responses in otherwise unresponsive neonatal animals. Mature macrophages were found to interfere with the production of immunological tolerance in neonatal animals and this feature was used to promote resistence to tumors caused by the vertically transmitted mammary tumor virus (MTV) in mice, which ordinarily induces immunologic tolerance in neonatal mice. In all 13 of 110 patients with the immune deficiency studied at the National Institutes of Health have developed malignancies. A number of different mechanisms have been shown to produce the functional defects in cell-mediated immunity that are present in all of these patients.

Anergy and defective in vitro lymphocyte transformation responses are characteristic of a number of immune deficiency disorders and/or malignant disease. To determine if defective macrophage lymphocyte interactions could account for the anergy, in vitro studies of these interactions were performed. The cooperation of macrophages is essential for responses of immune lymphocytes to specific antigens to occur. About 1 macrophage per 10 lymphocytes was found to be optimal for specific antigen induced blastogeneous to occur. Allogeneic macrophages could successfully cooperate with lymphocytes for these in vitro reactions. It was found that macrophages from anergic subjects were generally just as effective in reconstituting the responsiveness of normal purified lymphocytes as were normal macrophages. By contrast, normal macrophages were unable to reconstitute or promote responses by lymphocytes from anergic patients demonstrating that the deficient in vitro lymphocyte response in anergy could not be explained by defective macrophage function in vitro.

In other studies using new techniques developed in this laboratory patients with sprue were shown to manifest a marked increase in the rate of local gastrointestinal IgA and IgM synthesis following exposure to gliaden. It was further shown that the majority of newly formed IgA and IgM molecules represented antibody to this protein. Recently an in vitro model of gluten-sensitive enteropathy was developed in order to analyze the role of immunologic factors in this disease. Jejunal biopsy specimens were established in culture for as long as 48 hours. It was observed that alkaline phosphatase enzyme activity increased 1-2 fold in specimens from normal or diseased control individuals. In contrast, alkaline phosphatase activity in specimens from patients with gluten induced enteropathy increased 4-5 fold, and entered or approached the normal range of values. Concimitant with this biochemical improvement, electron microscopic studies showed morphologic improvement: increase in number and length of microvilli. When specimens were cultured in the presence of gliaden, improvement in enzyme activity was reduced or absent in specimens from patients with active GSE whereas gliaden had no effect on normal or control specimens. This inhibition in the recovery of enzyme activity by gliaden constitutes an in vitro model of gluten sensitive enteropathy.

The direct toxic potential of gliaden on jejunal mucosa was studied using the above in vitro model of GSE. Jejunal specimens obtained from patients in remission were cultured in the presence and absence of gliaden. It was found that enzyme activities rose in both circumstances. When the same group of patients were given a 7-10 day in vivo gluten challenge and specimens again obtained the cultured enzyme activity rose in the absence of gliaden but not in the presence of gliaden. These findings indicated that the toxic effect of gliaden in vitro was only manifested after a period of in vivo gluten challenge; i.e., it was necessary to activate an endogenous mechanism, the immunologic process, before gliaden could exert its effect. Such studies suggest that abnormal immune response to gliaden may be of significance in the pathogenesis of the sprue syndrome. Another very interesting finding in patients with gluten sensitive enteropathy is the fact that 89% of the patients have the histocompatibility antigen HL-A8. This contrast with a 22-24% incidence of HL-A8 among normal individuals. This suggests that the immune response to gluten may be due to the presence of a pathogeneic immune response gene which allows them to mount immunologic responses to gluten within the gastrointestinal mucosa. This is suggested by the fact that Ir genes in experimental animals are linked to histocompatibility genes. Dermatitis herpitiformis (DH) is a skin disease associated with various immunologic abnormalities. Some two-thirds of patients with this condition have a gastrointestinal lesion similar to that seen in GSE. The HL-A8 type of DH patients was determined and it was found that 59% had HL-A8. Thus, the HL-A8 histocompatibility gene is found in both patients with GSE and DH and may be the genetic link between the two conditions.

Detection of Circulating Tumor Related Antigens

A new double antibody radioimmunoassay for α-fetoprotein has been developed. This assay is sensitive to 5 ng of α-fetoprotein/ml of plasma. Eighty percent of patients with hepatomas, and 85% of patients with teratomas of the ovary and testes and a small percentage of patients with gastric and pulmonary tumors have markedly elevated α-fetoprotein levels. All patients with ataxia

telangiectasia, rare patients with subacute necrosis of the liver and pregnant women also have slightly elevated α-fetoprotein levels. Patients with other benign conditions had normal serum α-fetoprotein levels. Thus, this sensitive test has been of value in the detection of patients with hepatomas and teratomas of the ovary and testes. It has been of even greater value in following the efficacy of surgical treatment or chemotherapy of these tumors. The detection of α-fetoprotein in the serum in treated patients reflecting a few residual tumor cells was possible with this method when α-fetoprotein was undetectable as assessed by conventional tests. On the basis of the results of the conventional tests the therapy had been stopped in these patients. The tumors, however, recurred in all cases where the α-fetoprotein assay remained positive by the radioimmunoassay test.

A new double antibody radioimmunoassay for the immunoglobulin IgE has been established. Using this assay it was shown that patients with Hodgkin's disease have markedly elevated IgE levels in their serum. Patients with tumors of the B-cell lymphocyte system, i.e., patients with IgA and IgG myelomas with macroglobulinemia Waldenström and especially those with chronic lymphocytic leukemia have markedly reduced serum IgE levels. The majority of patients with chronic lymphocytic leukemia had IgE levels in the serum that were undetectable, i.e., less than 2% of normal. The depression of IgE in these patients was much more profound than the depression of any of the other immunoglobulin classes. Markedly depressed IgE levels were also demonstrated in patients with sex-linked agammaglobulinemia, idiopathic acquired hypogammaglobulinemia and thymoma and hypogammaglobulinemia. In addition IgE levels were depressed in 80% of patients with ataxia telangiectasia and 28% of the patients studied with isolated IgA deficiency. The IgE levels were normal in patients with hypogammaglobulinemia due to a shortened survival of immunoglobulin molecules, i.e., in patients with myotonic dystrophy, gastrointestinal protein loss, and patients with familial hypercatabolic hypoproteinemia. The IgE levels were elevated in patients with the Wiskott-Aldrich syndrome and with parasitism. Using radioiodinated IgE turnover studies the abnormalities of IgE levels in disease were shown to be due to differences in rates of synthesis of these molecules rather than disorders of their catabolic pathways.

Bilirubin Metabolism

Studies of hepatic and erythrocyte physiology and pathophysiology were continued using the techniques developed in this laboratory for the measurement of hepatic clearances of various substances in man. These studies involve analysis of the rates of bilirubin production and the mechanism by which the liver excretes bilirubin. While the bulk of daily bilirubin production is derived from the hemoglobin of red blood cells, a discrete fraction is of a non-erythroid origin. By mathematical analysis of the relationships between bilirubin production, total red cell volume and red cell life span, a new approach to quantitation of the non-erythropoietic component of bilirubin production was derived. These studies indicate a non-erythropoietic component of 0.3 - 1.0 mg of bilirubin/kg/day, representing 15-30% of total bile pigment production in normal man. These values were confirmed by direct measurement of hepatic bilirubin production in patients with acute intermittent porphyria.

Further studies of the effects of glutethimide and phenobarbital on various aspects of hepatic function have indicated that: (1) drug therapy produces no changes in total heme turnover; however, (2) plasma bilirubin turnover falls, suggesting a change in the routes of bilirubin elimination; (3) drug therapy produces a marked increase in hepatic bilirubin clearance in patients with Gilbert's syndrome, but little change in normal volunteers.

By means of detailed studies of BSP kinetics, it has been possible to divide patients with Gilbert's syndrome into three subgroups, one of which has normal BSP metabolism, one defective hepatic BSP uptake, and the third, a defect in a later stage of BSP metabolism. These data clearly indicate the heterogeniety of patients classified as having Gilbert's and suggest that multiple mechanisms may be responsible for the hyperbilirubinemia in this condition.

Porphyrin Metabolism

Acute intermittent porphyria has been characterized biochemically by the increased urinary excretion of the porphyrin precursors δ-aminolevulinic acid and porphobilinogen. This was shown to be due to a marked genetic increase in hepatic ALA synthetase. A partial block in heme synthesis may relate to the observed induction of hepatic δ-aminolevulinic acid synthetase (ALAS), the end product heme having been shown to repress the synthetase production. Because of this consideration hematin was administered intravenously to a patient with a severe attack of acute intermittent porphyria. This caused the diminution of serum δ-aminolevulinic acid and porphrobilinogen to normal in the presence of complete renal failure. The "cure" of the biochemical abnormalities of porphyria was evident when a number of other forms of therapy were shown to have no effect on the disease of this patient. Thus, this use of hematin based on theoretical considerations may be of importance in the repression of the over-production of certain porphyrin precursors in patients with acute attacks of acute intermittent porphyria. In other clinical studies in patients with acute intermittent porphyria, such patients have been shown to have evidence of diabetes insipidus long after an acute attack of the disease has subsided. In other studies the hypertension of acute intermittent porphyria has been shown to be exquisitely sensitive to the B-adrenergic blocker, propranolol.

The mechanism by which glucose blocks the induction of hepatic ALA synthetase ("glucose effect") has been studied extensively. The mechanism is proving to be complex, and although it has not been elucidated, a number of significant findings are now evident. In bacteria other laboratories have shown that the synthesis of a number of enzymes which are subject to the glucose effect are stimulated by cyclic AMP, which reacts with a specific protein to form a complex which augments activity of the promoter locus. In these organisms glucose acts by lowering cyclic AMP levels. No role of cyclic AMP could be shown in the synthesis of hepatic ALA synthetase. Both hepatic ALA synthetase and tyrosine aminotransferase were shown to be subject to a glucose effect, which operates on the two enzymes by different mechanisms. Experimental diabetes partially blocks the induction of hepatic ALA synthetase, as does 2-deoxyglucose, although the later is a potent inducer of tyrosine aminotransferase and augments the induction of this enzyme by other agents.

Diazoxide, an agent used to produce hyperglycemia, also inhibits the induction of hepatic ALA synthetase. Allylisopropylacetamide (AIA), an agent used to produce experimental porphyria, produces a profound synergistic effect on the hyperglycemia caused by diazoxide. AIA produces no hyperglycemia by itself. This synergistic effect on diazoxide induced hyperglycemia has now been shown to occur with certain barbiturates also. These studies have clearly shown that there are different mechanisms for the glucose effect on different enzymes in mammalian liver and that some of these do not involve cyclic AMP.

Calcium Metabolism

Studies on the effects of diphosphonates in a variety of bone diseases have been continued. Using Ca^{47} kinetics and balance techniques it was shown that diphosphonates can decrease rates of bone turnover in a variety of patients and that ectopic calcifications in a variety of diseases, e.g., calcinosis and myositis ossificans progressiva, can be mobilized by the drug.

Studies of the mechanisms of calcium absorption in man have been directed toward defining the absorption mechanisms as a function of gastrointestinal lumenal calcium concentrations so that one can effectively diagnose and treat patients with calcium malabsorptions. Using the oral Ca^{47} administration techniques together with computer deconvolution, we have been able to define a saturable curve for calcium absorption as a function of oral calcium load. There appears to be two components in this calcium absorption curve. One components saturates at a calcium load of about 200 mg whereas another component does not reach saturation at a calcium load of even 600 mg. The second component is probably due to diffusion. We are now studying patients with various calcium malabsorptions to define which component is altered by vitamin D or parathyroid hormone treatment.

Amino Acid Transport

Studies of the mechanism of amino acid transport in the kidney have emphasized the interaction of cyclic AMP with the sodium potassium ATPase. Cyclic AMP stimulates amino acid transport; the stimulation is dependent on sodium ion, is inhibited by ouabain and is abolished by inhibitors of protein synthesis. We have, therefore, concentrated on cyclic AMP mediated alterations in sodium potassium ATPase isolated from rat kidney cortex. A brush border preparation (P_2) as well as a membrane fraction free of brush borders (S_3) were isolated. The sodium potassium ATPase from each fraction was then studied using a variety of techniques. The activation of the enzyme by sodium and potassium in both fractions appears to be similar. We found, however, that there was a difference between P_2 and S_3 relative to a cyclic AMP mediated sodium potassium ATPase in S_3 which is more sensitive to phosphate inhibition. This alteration may allow for an accumulation of high energy phosphate enzyme intermediates which may be used for amino acid transport.

Studies on the regulation of amino acid transport in bone cells using fetal rat calvaria have been continued. Previous work from our laboratory showed that the amino acids transported by sodium-dependent mechanisms are under cyclic AMP and insulin regulation. We, therefore, emphasized the regulatory

mechanisms of the sodium-dependent transport mechanisms for both α-aminoisobutyric acid and proline. Inhibitors of protein synthesis inhibited the transport of α-aminoisobutyric acid, proline and hydroxyproline, but had no effect on the active uptake of all other amino acids tested. Furthermore, only the sodium sensitive transport component for α-aminoisobutyric acid was affected by puromycin. The half life of disappearance of the sodium dependent transport mechanism with puromycin incubation appeared to be about 40 minutes, suggesting that a half life of a component of the sodium transport mechanism is of this magnitude. Another mechanism for regulation of amino acid transport. depends on the intracellular concentration of specific amino acids. Preloading cells with 10 mM glycine, proline or hydroxyproline greatly inhibited the active uptake of AIB or proline. Preloading cells with all other amino acids had no effect on the apparent k_m of the transport mechanism for AIB. Furthermore, there were some definite differences in the lag times produced by these two regulatory mechanisms. The time course of the puromycin effect was exponential suggesting a turnover of a component of the transport mechanism. In contrast, the effects of amino acid loading were much more rapid and showed a plateau suggesting that intracellular amino acids could saturate carrier sites, thus, inhibiting the active influx of amino acid. These studies suggest that a feedback inhibition of amino acid transport mechanism exists.

Serial No. NCI-4002(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of the Physiology and Pathophysiology of Plasma Protein Metabolism

Previous Serial Number: SAME

Principal Investigator: Thomas A. Waldmann, M.D.

Other Investigators: Warren Strober, M.D., R. Michael Blaese, M.D. and William Terry, M.D.

Cooperating Units: Immunology Branch, NCI

Man Years

Total: 3
Professional: 1
Others: 2

Project Description

Objectives: The objectives of the study were to determine the normal rate of synthesis, transport and catabolism of the plasma proteins, especially the immunoglobulins. To determine the physiological factors controlling these metabolic parameters and to determine the pathophysiology of disorders of serum protein metabolism in patients with neoplastic disease and in patients with hereditary disorders of immunoglobulin metabolism. In addition, studies were directed toward developing techniques for the detection of circulating tumor-related antigens and in determining the metabolism of these protein antigens.

Methods Employed: The rates of synthesis, catabolism and distribution of the serum proteins were studied using radioiodinated labeled purified proteins. The purified proteins were labeled *in vitro* with ^{131}I or ^{125}I by the iodine monochloride or the lactoperoxidase techniques. The curves of radioactivity in the urine, stool, serum, nasal, and salivary secretions were used in mathematical multicompartmental models to determine the rates of protein synthesis, catabolism and distribution into the extravascular compartments and external secretions. In addition, sucrose gradient ultracentrifugation as well as affinity chromatography were used as new techniques for the demonstrations of IgG receptors on the surface of cells. New double antibody radioimmunoassays were established to detect α-fetoprotein and IgE immunoglobulin.

Major Findings: A new double antibody radioimmunoassay for α-fetoprotein has been developed. This assay is sensitive to 5 ng of α-fetoprotein/ml of plasma. Eighty percent of patients with hepatomas, and 85% of patients with teratomas of the ovary and testes and a small percentage of patients with gastric and pulmonary tumors have markedly elevated serum α-fetoprotein levels. All patients with ataxia telangiectasia studied, rare patients with subacute necrosis of the liver, as well as all pregnant women also have slightly elevated α-fetoprotein levels. Patients with other benign conditions had normal serum α-fetoprotein levels. Thus, this sensitive test has been of value in the detection of patients with hepatomas and teratomas of the ovary and testes. It has been of even greater value in following the efficacy of surgical treatment or chemotherapy of these tumors. The detection of α-fetoprotein in the serum in treated patients, reflecting a few residual tumor cells, was possible with this method when α-fetoprotein was undetectable as assessed by conventional tests. On the basis of the results of the conventional tests the therapy had been stopped in these patients. The tumors, however, recurred in all cases where the α-fetoprotein assay remained positive by the radioimmunoassay test.

A new double antibody radioimmunoassay for the immunoglobulin IgE has been established. Normal individuals had a geometric mean IgE concentration of 105 ng/ml. The fraction of the intravenous pool of IgE catabolized per day as assessed with ^{125}I IgE turnover studies was 72%. Normal individuals synthesized 30 ng of IgE/kg/day. Using the radioimmunoassay, for IgE it was shown that patients with Hodgkin's disease have a 15-fold increase in their serum IgE levels. Large groups of patients with tumors of the B-cell (thymic independent) lymphocyte system i.e., patients with IgA and IgG myelomas, patients with macroglobulinemia of Waldenström and especially those with chronic lymphocytic leukemia have markedly reduced IgE levels. The majority of patients with chronic lymphocytic leukemia had IgE levels in the serum that were undetectable, i.e., less than 2% of normal. The depression of IgE in these patients with chronic lymphocytic leukemia was much more profound than the depression of any of the other immunoglobulin classes. All of the patients with a generalized reduction in immunoglobulin production associated with an immunological deficiency disease had a markedly reduced level of IgE, i.e., all patients with Bruton-type agammaglobulinemia, with idiopathic acquired hypogammaglobulinemia or with thymoma and hypogammaglobulinemia had IgE levels in the serum that were undetectable or were less than 10% of normal. IgE deficiency was also detected in a large number of patients with different forms of dysgammaglobulinemia, i.e., disorders where there is a reduced synthesis of some classes of immunoglobulins but with normal synthesis of others. For example, 80% of patients with ataxia telangiectasia were IgE deficient. Sixty-six percent of these patients were also IgA deficient, and 57% had combined IgE and IgA deficiency. Eleven of 25 individuals studied with isolated IgA deficiency were also IgE deficient. It is of interest to note that all 11 patients with both IgA and IgE deficiency were uniformly asymptomatic. However, there was an exceedingly

high incidence (71%) of respiratory disease in the 14 IgA deficient individuals who were not IgE deficient. These data fail to support the concept of a protective role for IgE in respiratory tract immunity. They suggest, rather, that the IgE may play a role in the pathogenesis of respiratory tract disease in IgA deficient patients. The IgE levels in groups of patients with hypogammaglobulinemia secondary to a shortened immunoglobulin survival were found to be normal in contrast to the markedly reduced IgE levels found in patients with a generalized defect in immunoglobulin synthesis, i.e., the IgE concentration in patients with myotonic dystrophy and a low IgG concentration and a short IgE survival did not differ significantly from normal, nor was it depressed in the patients with familial hypercatabolic hypoproteinemia and a shortened survival of many proteins. The serum IgE levels were also normal in the 10 patients who had intestinal lymphangiectasia and a generalized loss of serum proteins into the gastrointestinal tract. Thus, the determination of the serum IgE level is of value in differentiating hypogammaglobulinemia secondary to decreased synthesis from that secondary to a short immunoglobulin survival. The IgE levels were markedly elevated in patients with the Wiskott-Aldrich syndrome. It has been suggested that patients with the Wiskott-Aldrich syndrome have a defect in the afferent limb of immunity with an inability to process and recognize these antigens normally. The high IgE levels observed in these patients may reflect chronic stimulation of the IgE synthesizing system by antigens that cannot be adequately processed and eliminated by these patients. The IgE levels were markedly elevated in patients with parasitism with a mean level of IgE in the serum over 100 times normal in patients with schistosomiasis. These studies suggest that IgE may play a role in the host defenses against parasitic infestation.

Studies of the transport of serum proteins across the gastrointestinal tract of neonatal rats were used as a model to study the mechanism of transcellular proteins and to study one factor controlling the rate of protein catabolism. Uptake of intestinal cells and transcellular transport of protein were shown to be saturable processes that are specific for IgE molecules. The first step in the intestinal transport of IgG involves the formation of a high molecular weight complex between these molecules and an IgG specific receptor that can be demonstrated on purified enterocyte microvillus membranes. The Fc piece is the submolecular region of the IgG molecule that is required for interaction with the cell surface receptor and for specific uptake and transport of the whole molecule. In related studies, the formation of IgG cell membrane complexes was also demonstrated in carcas homogenates following intravenous administration of radioiodinated IgG in germ free mice. These mice have a low IgG concentration and a low IgG survival because of the IgG concentration-catabolism effect. Thus, it would appear that a major process involved in both the specific IgG transport between the mother and neonatal rat and the IgG concentration-catabolism effect may involve competition for a limited number of saturable receptors on cell membranes that are specific for the IgG molecule. Such receptors would protect the immunoglobulin from catabolism and would be necessary for the specific cellular

uptake and transport of this molecule.

Significance to Biomedical Research: The detection of circulating tumor-related antigens is of significance in the diagnosis of neoplastic disease and is of special relevance in evaluating the activity of the neoplastic disease and determining the efficacy of therapy of these disorders. The studies on immunoglobulin metabolism extend our knowledge of the normal metabolism and physiological control mechanisms that affect immunoglobulin synthesis, transport and catabolism. They are of importance in understanding the pathophysiology of disorders of immunoglobulin levels in patients with disease.

Proposed Course: Studies directed toward understanding the pathways and controlling mechanisms of the synthesis, transport, and catabolism of serum protein will be continued. Increasing emphasis will be placed on the development of new techniques for detection of circulating tumor-related antigens. Studies will also be directed toward defining the synthetic rates and rates of catabolism of such tumor-related antigens.

Honors and Awards

None

Publications

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Serial No. NCI-4003(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of Porphyrin Metabolism in the Tumor-Bearing Host and Porphyria

Previous Serial Number: SAME

Principal Investigator: Donald P. Tschudy, M.D.

Other Investigators: Joel Levine, M.D. and Annie Collins

Cooperating Units: Laboratory of Parasitic Diseases, NIAID, Virus and Disease Modification Section, Viral Leukemia and Lymphoma Branch, NHLI.

Man Years

Total: 3½
Professional: 2
Others: 1½

Project Description:

Objectives: To study the mechanisms controlling heme biosynthesis and biochemical and clinical aspects of the porphyrias. To apply the knowledge gained from the above objectives to the study of tumors and tumor-host relationships.

Methods Employed: (A) Quantitative enzyme determinations. (B) Chemical determination of porphyrins and porphyrin precursors. (C) Use of tissue homogenates, slices and mitochondria in studying metabolic pathways.

Major Findings: The mechanism by which glucose blocks the induction of hepatic ALA synthetase ("glucose effect") has been studied extensively. The mechanism is proving to be complex, and although it has not been elucidated, a number of significant findings are now evident. In bacteria other laboratories have shown that the synthesis of a number of enzymes which are subject to the glucose effect is stimulated by cyclic AMP, which reacts with a specific protein to form a complex which augments activity of the promotor locus. In these organisms glucose acts by lowering cyclic AMP levels. No role of cyclic AMP could be shown in the synthesis of hepatic ALA synthetase. Both hepatic ALA synthetase and tyrosine aminotransferase were shown to be subject to a glucose effect, which operates on the two enzymes by different mechanisms. Experimental diabetes partially blocks the induction of hepatic ALA synthetase, as

does 2-deoxyglucose, although the latter is a potent inducer of tyrosine aminotransferase and augments the induction of this enzyme by other agents. Diazoxide, an agent used to produce hyperglycemia, also inhibits the induction of hepatic ALA synthetase. Allylisopropylacetamide (AIA), an agent used to produce experimental porphyria, produces a profound synergistic effect on the hyperglycemia caused by diazoxide. AIA produces no hyperglycemia by itself. This synergistic effect on diazoxide induced hyperglycemia has now been shown to occur with certain barbiturates also. These findings should be investigated for possible clinical application to the treatment of refractory hypoglycemia. The studies have clearly shown that there are different mechanisms for the glucose effect on different enzymes in mammalian liver and that some of these do not involve cyclic AMP.

Hematin has been administered intravenously to a patient with acute intermittent porphyria and was shown to bring the blood levels of the porphyrin precursors down to normal in the presence of complete renal failure.

This "cure" of the biochemical abnormalities was evident when a number of other forms of therapy were shown to have no effect on the disease in this patient. The use of hematin in this disease was based on theoretical considerations and the fact that it produces a temporary biochemical remission answers certain questions about the disease.

Some patients with acute intermittent porphyria have been shown to have evidence of mild diabetes insipidus long after an acute attack of the disease has subsided.

The hypertension of acute intermittent porphyria in some patients has been exquisitely sensitive to the β-adrenergic blocker, propranolol.

Significance to Biomedical Research: Mechanisms of the control of metabolic pathways and the synthesis of enzymes in normal and diseased tissued are being elucidated. New abnormalities of control mechanisms in tumors have been demonstrated and will be studied further.

Proposed Course: (A) To continue studies of the factors controlling heme and heme-enzyme synthesis in normal and pathologic tissues. (B) To continue studies of biochemical abnormalities of acute porphyria in man and experimental animals. (C) To study control mechanisms of heme synthesis in tumors and the livers of tumor-bearing hosts.

Honors and Awards

Superior Service Award

Publications

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Serial No. NCI-4004(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of Amino Acid Transport and Amino Acid Biosynthesis

Previous Serial Number: SAME

Principal Investigator: James M. Phang, M.D.

Other Investigators: David L. Valle, M.D.

Cooperating Units: None

Man Years:

Total: 6
Professional: 2
Others: 4

Project Description:

Objectives: Cell membrane may be an important component in the malignant transformation of cells. This project emphasizes the membrane active transport of amino acids and regulatory mechanisms which alter the transport process. Since protein synthesis is altered by rates of amino acid transport, regulation of amino acid transport may control growth rates and proliferation of normal and malignant cells. This project is relevant to objective 3, approaches 1 and 5 of the National Cancer Program.

Methods Employed: A variety of biochemical techniques have been utilized to study amino acid transport processes in bone cells and kidney and amino acid biosynthesis in liver cells. Uptake of amino acids may be assessed by using labeled L-amino acids and rates of protein and collagen synthesis can be obtained by incubating tissues with L-proline C^{14} and separation of the radioisotopically incorporated amino acids as proline and hydroxyproline by thin layer chromatography. Our kidney cell membrane fractions are isolated and purified using discontinuous sucrose density gradients. The activity of sodium potassium ATPase as well as other membrane enzymes are assayed. The phosphoenzyme intermediates of the sodium potassium ATPase are isolated using Millipore filter techniques. The enzymes for biosynthesis of proline in the liver are studied using labeled precursors such as L-ornithine and L-glutamic acid and by isolating the products using ion-exchange chromatography.

Major Findings: Our studies on the regulatory mechanisms in amino acid transport and amino acid biosynthesis has been in three major areas (1) the biochemical mechanisms of amino acid transport in the kidney cortex, (2) mechanisms of regulation and turnover of transport mechanisms in bone cells and (3) mechanisms of regulation of proline biosynthesis in mammalian cells.

Our studies on the mechanisms of amino acid transport in the kidney have emphasized the interaction of cyclic AMP with the sodium potassium ATPase. Cyclic AMP stimulates amino acid transport; the stimulation is dependent on sodium ion, is inhibited by ouabain and is abolished by inhibitors of protein synthesis. We, therefore, concentrated on cyclic AMP mediated alterations in sodium potassium ATPase isolated from rat kidney cortex. Our isolation procedures included discontinuous sucrose density gradients and treatment with deoxycholate. Using these methods we isolated a brush border preparation (P_2) as well as a membrane fraction free of brush border (S_3). We then studied the sodium potassium ATPase from each fraction using a variety of techniques. We first examined the sodium potassium activity in both of these fractions and found that the activation of the enzyme by sodium and potassium in both fractions appears to be similar. We found, however, that there was a difference between P_2 and S_3 relative to a cyclic AMP mediated sodium potassium ATPase in S_3 which is more sensitive to phosphate inhibition. This alteration may allow for an accumulation of high energy phosphate enzyme intermediates which may be used for amino acid transport. We are now studying the formation of phosphoenzyme intermediates using a Millipore filter technique to recover the phosphorylated enzyme.

We have continued our studies of the regulation of amino acid transport in bone cells using fetal rat calvaria. Previous work from our laboratory showed that the amino acids transported by sodium-dependent mechanisms are under cyclic AMP and insulin regulation. We, therefore, emphasized the regulatory mechanisms of the sodium-dependent transport mechanisms for both alpha-aminoisobutyric acid and proline. We found that both puromycin and cycloheximide as well as amino acid loading inhibited the sodium dependent transport mechanisms for alpha-aminoisobutyric acid. Inhibitors of protein synthesis inhibited the transport of alpha-aminoisobutyric acid, proline and hydroxyproline, but had no effect on the active uptake of all other amino acids tested. Furthermore, only the sodium sensitive transport component for alpha-aminoisobutyric acid was affected by puromycin. The half life of disappearance of the sodium dependent transport mechanism with puromycin incubation appeared to be about 40 minutes, suggesting that a half life of a component of the sodium transport mechanism is of this magnitude. Another mechanism for regulation of amino acid transport depends on the intracellular concentration of specific amino acids. We found that preloading cells with 10 mM glycine, proline or hydroxyproline greatly inhibited the active uptake of AIB or proline. Preloading cells with all other amino acids had no effect on either AIB or proline transport. Both puromycin and

amino acid loading decreased transport V_{max} but had no effect on the apparent k_m of the transport mechanism for AIB. Furthermore there were some definite differences in the lag times produced by these two regulatory mechanisms. The time course of the puromycin effect was exponential suggesting a turnover of a component of the transport mechanism. In contrast, the effects of amino loading were much more rapid and showed a plateau suggesting that intracellular amino acids could saturate carrier sites thus inhibiting the active influx of amino acid. These studies suggest that a feedback inhibition of amino acid transport mechanism exists.

We have studied the enzymatic pathways by which proline may be synthesized in a rat liver. The methodology for the enzyme assay includes the separation of the product amino acids on a dowex column eluted with 1N hydrochloric acid. Using this column we can separate glutamic acid, glutamic semialdahyde and proline. The enzyme we have concentrated on is the transaminase which catalyzes the formation of glutamic semialdehyde from L-ornithine and α-ketoglutarate. Glutamic semialdahyde is in spontaneous equilibrium with pyrroline-5-carboxyclic acid. The latter compound is then enzymatically reduced in the presence of DPNH or TPNH to proline. In addition, pyrroline-5- carboxylic acid may be oxidized in the presence of DPN or TPN to glutamic acid. Our studies thus far suggests that the reductase and the dehydrogenase may be regulated in rat liver. Transaminase activity on the other hand appears to be unchanged with hormonal and physiologic perturbations.

Significance to Biomedical Research: Cell membranes may play an important role in the regulation of cellular physiology. Changes in cell membrane function affects cellular metabolism and cell proliferation. Some of these changes are produced by hormone regulation as a component of normal control mechanisms. Cyclic AMP appears to the intermediate by which these hormones exert their action on transport. Abnormal changes in membrane function may result in uncontrolled neoplastic cell growth. Some investigators have proposed that one of the primary changes produced by malignant transformation may be alterations in membrane function. These workers have shown that a viral transformation of cells can increase rates of sugar and amino acid transport even prior to increases in cell growth rates. Our studies on the regulation of amino acid transport may elucidate the biochemical mechanisms of membrane function as well as derangements of these functions in malignant cells.

Proposed Course: We will continue our studies on the cyclic AMP mediated changes in sodium potassium ATPase from the kidney cortex. We hope to refine our techniques in obtaining a membrane fraction which will contain the cyclic AMP sensitive sodium potassium ATPase free of other contaminating membrane fractions. Using the Millipore filter technique to isolate phosphoenzyme intermediates, we hope to show biochemical interactions between cyclic AMP activated protein kinases with the sodium potassium ATPase and alterations in phosphorylation of carrier proteins which mediate amino acid transport.

Our studies in fetal rat calvaria showing stimulation by insulin and cyclic AMP, inhibition by inhibitors of protein synthesis and regulation by intracellular amino acids may all be used to define abnormal transport mechanisms in malignant cells. We are extending our studies of amino acid transport to normal fibroblasts and to cell lines which have undergone malignant transformation.

Honors and Awards

None

Publications

Hahn, T.J., Downing, S.J. and Phang, J.M.: Insulin effect on amino acid transport in bone: dependence on protein synthesis in sodium. Amer. J. Physiol. 220: 1717-1723, 1971.

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Weiss, I.W., Harris, S., Downing, S.J., and Phang, J.M.: A micro-method for determination of phosphoenzyme intermediated of sodium potassium ATPase. Anal. Biochem. In press.

Phang, J.M. and Downing, S.J.: Amino acid transport in bone: Regulation by cyclic AMP. Submitted to Amer. J. Physiol.

Serial No. NCI-4009(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: A Study of the Factors Controlling Red Blood Cell Synthesis and Destruction

Previous Serial Number: SAME

Principal Investigator: Paul D. Berk, M.D.

Other Investigators: Terrence F. Blaschke, M.D.

Cooperating Units: None

Man Years:

Total: 5
Professional: 5
Others: 0

Project Description:

Objectives: To study the factors influencing rates of red blood cell production and destruction in normal and pathological states in man, the dog and rat, with particular emphasis on the role of ineffective erythropoiesis in the overall body economy of heme. Studies of hepatic heme enzyme turnover and organic anion transport are also conducted as an outgrowth of the use of isotopically labeled bilirubin to quantitate erythropoiesis.

Methods Employed: Erythropoiesis is quantitated by the following techniques whole blood and red cell volumes are determined with ^{51}Cr-labeled red blood cells; red cell life span is determined directly from either ^{14}C-glycine incorporation or tritiated diisopropylfluorophosphate labeling, or calculated from plasma bilirubin turnover; plasma and red blood cell iron turnovers are calculated from the plasma clearance of ^{59}Fe-labeled transferrin, and subsequent incorporation of radio-iron into circulating erythrocytes. Ineffective erythropoiesis is estimated from the incorporation of ^{14}C label into fecal urobilinogen during the first ten days after IV administration of ^{14}C-glycine, as well as by computer analysis of the specific activity curve of plasma bilirubin following the administration of labeled heme precursors such as glycine and delta-aminolevulinic acid. Bilirubin-^{14}C and bilirubin-^{3}H are prepared biosynthetically from delta-aminolevulinic acid; bilirubin production rate, hepatic bilirubin clearance and storage, and intercompartmental rate constants in the

bilirubin excretory pathway are determined by computer analysis of the plasma radio-bilirubin disappearance curves. Hepatic transport of organic anions such as sulfobromophthalein (BSP) and indocyanine green is studied by computer analysis of their respective plasma disappearance curves.

Major Findings:

Measurement of bilirubin production and red cell life span -- We have previously demonstrated that daily bilirubin production could be accurately determined from the plasma disappearance curve of intravenously injected unconjugated radiobilirubin. If total red cell volume was simultaneously measured, mean red cell life span could be calculated by a simple formula. Although this technique is more rapid than any of the currently available methods for determination of red cell survival, its general application is limited by the need for a computer to analyze the plasma bilirubin curves. In the past year we have demonstrated that bilirubin production could also be accurately calculated from the specific activity of a single urobilin sample isolated from a pool of feces following injection of radio-bilirubin. This should significantly increase the utility of this approach to the calculation of red cell life span in clinical situations.

Studies of non-erythropoietic sources of bilirubin -- Following administration of isotopically labeled delta-aminolevulinic acid, labeled unconjugated bilirubin circulates in the plasma. This labeled bilirubin is formed almost exclusively in the liver. Mathematical techniques have been developed by which the amount of isotopically labeled precursor which has been incorporated into bilirubin can be calculated. Studies have been done in seven normal individuals, two patients with red cell aplasia, six patients with Gilbert's syndrome, and three patients with acute intermittent porphyria. In the porphyrics the actual amount of bilirubin formed by the liver was calculated by application of the labeled precursor-product relationship to the specific activity of urinary PBG. In porphyrics hepatic bilirubin production ranged from 0.4 - 0.9 mgm/kg/day. Since we have also showed that hepatic bilirubin production in porphyric patients is not increased, similar figures may be applicable to normal subjects.

Simultaneous measurement of BRP, TRCV and mean red cell survival using DFP-^{3}H (LS_{DFP}) has made possible a new approach to the study of ineffective erythropoiesis and other non-erythroid sources of bile pigment. Bilirubin production from red cells was estimated from TRCV and LS_{DFP}, and the result compared with values for total bilirubin production. The non-erythroid fraction of bilirubin production was found to consist of a constant component C of about 0.9 mgm/kg/day and a second component proportional to the erythropoietic rate (proportionality constant k = .07). The value of C was thus similar to the value for hepatic bilirubin production as directly calculated in porphyric subjects. From the values of

BRP, C and k, it was possible to estimate the early labeled peak, which averaged 30% of BRP in normal subjects. At accelerated rates of erythropoiesis, the early labeled peak increased in absolute magnitude but decreased when expressed as a fraction of total BRP, averaging 15% when red cell survival was less than 40 days.

In a related approach to this problem, data for bilirubin production were plotted against total red cell volume (TRCV) in 45 patients in whom ^{51}Cr red cell survival studies were normal. The patient population included normal volunteers, and patients with acute intermittent porphyria, polycythemia vera and Gilbert's syndrome, in whom TRCV ranged from 15-41 ml/kg. The Y intercept of the regression line was taken as a measure of non-erythropoietic bilirubin production, and ranged from 0.3 - 1.0 mg/kg/day.

Studies of the effects of drugs on bilirubin production -- The effects of either phenobarbital or glutethimide on the rate of heme turnover has been measured by studies of carbon monoxide and bilirubin production in normal volunteers and patients with Gilbert's syndrome. In contrast to the limited data in the literature, we have found no drug induced changes in total body heme turnover - as measured by CO production - in more than 30 patients and normal volunteers. While CO production was unchanged, plasma bilirubin turnover actually fell during drug administration, suggesting stimulation of pathways for bilirubin excretion which bypass the plasma. The fall in plasma bilirubin concentration observed in normal volunteers during drug therapy results almost entirely from decreased plasma bilirubin turnover. The similar change in patients with Gilbert's syndrome is due in part to this phenomenon, but also result from a significant increase in hepatic bilirubin clearance not observed in normal volunteers.

Studies of hepatic function in Gilbert's syndrome -- Detailed studies of BSP kinetics in 18 patients with Gilbert's syndrome revealed that this entity could be subdivided into 3 groups. In approximately $\frac{1}{3}$ of patients, BSP metabolism was normal; in $\frac{1}{3}$ there was defective BSP uptake; and in $\frac{1}{3}$ there was an abnormality at a later stage of BSP transport. Since BSP is conjugated with glutathione, rather than glucuronic acid, these data do not support the concept that hyperbilirubinemia in Gilbert's syndrome results solely from defective hepatic UDP bilirubin-glucuronyl transferase. Presumably each of these 3 phenotypically similar subgroups results from a different lesion. Classification of patients into homogenous groups may simplify precise elucidation of the various lesions.

Significance to Biomedical Research: Studies with isotopically labeled bilirubin have produced the following useful results: (1) determination of the bilirubin production rate for a wide variety of disorders; (2) a new and rapid method for calculation of the mean red cell life span, both in normal individuals and individuals with hemolysis; (3) establishing of the normal range for the plasma unconjugated bilirubin concentration; (4) a method by which the hepatic clearance of bilirubin can be determined

at all plasma unconjugated bilirubin concentrations; (5) studies of hepatic bilirubin production in patients with porphyria have provided the first measurement of this parameter in man, and have led to further insights into the biochemical defect in this disease; (6) studies with Doriden® and phenobarbital are leading to a better understanding of the mechanisms by which the liver metabolizes drugs and responds to exogenous materials; (7) detailed studies of hepatic organic anion transport in Gilbert's syndrome are leading to an improved understanding both of this disorder and of normal hepatic function.

Proposed Course: Measurements of hepatic bilirubin production will be extended to include normals and patients with various liver diseases. The basic mechanisms underlying hepatic enzyme induction by drugs will be investigated. Further studies of organic anion excretion in Gilbert's syndrome will be pursued.

Honors and Awards

None

Publications

Bloomer, J.R., Berk, P.D., Bonkowsky, H.L., Stein, J.A., Berlin, N.I., and Tschudy, D.P.: Blood volume and bilirubin production in acute intermittent porphyria. New Eng. J. Med. 284: 17-20, 1971.

Bloomer, J.R., Berk, P.D., Howe, R.B., and Berlin, N.I.: Bilirubin metabolism in congenital nonhemolytic jaundice. Ped. Res. 5: 256-274, 1971.

Bloomer, J.R., Berk, P.D., Howe, R.B., and Berlin, N.I.: Interpretation of plasma bilirubin levels based on studies with radiobilirubin. J. Amer. Med. Assoc. 218: 216-220, 1971.

Jones, E.A., Bloomer, J.R. and Berlin, N.I.: The measurement of the synthetic rate of bilirubin from hepatic hemes in patients with acute intermittent porphyria. J. Clin. Invest. 50: 2259-2265, 1971.

Berk, P.D., Bloomer, J.R., Howe, R.B., Blaschke, T.F., and Berlin, N.I.: Bilirubin production as a measure of red cell life span. J. Lab. Clin. Med. 79: 364-378, 1972.

Berk, P.D., Blaschke, T.F. and Waggoner, J.G.: Defective BSP clearance in patients with constitutional hepatic dysfunction (Gilbert's syndrome). Gastroenterology. In press.

Berk, P.D. and Blaschke, T.F.: Detection of Gilbert's syndrome in patients with hemolysis, using radioactive chromium. Ann. Int. Med. In press.

Serial No. NCI-4013(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of Calcium Metabolism in Malignant Disease and Metabolic Diseases of Bone

Previous Serial Number: SAME

Principal Investigator: James M. Phang, M.D.

Other Investigators: David L. Valle, M.D. and Mones Berman, Ph.D.

Cooperating Units: Mathematical Research Branch, NIAMD

Man Years:

Total: 4
Professional: 2
Others: 2

Project Description:

Objectives: Osseous metastases with or without accompanying hypercalcemia are a frequent occurrence in neoplastic disease. In many patients the hypercalcemia can be life threatening. This project is designed to investigate physiologic and pathophysiologic regulatory mechanisms and to understand derangements of these mechanisms with malignant processes. This project is relevant to objective 3, approach 1 and objective 7, approach 2 of the National Cancer Program.

Methods Employed: Serum Ca^{47} disappearance and excretion of Ca^{47} into urine and feces are combined with metabolic balance data in a computer multicompartmental model to give steady state rate constants and numerical values for metabolic parameters. The physiological hypothesis suggested by this analysis are tested in animal and in vitro experiments and by additional studies of calcium metabolism in man. Furthermore, the uptake of Ca^{47} into the blood pool after an oral administered dose is used to define the absorption of calcium from the gastrointestinal tract. By varying the calcium load of the test dose we can define a quantitative relationship between calcium absorption and gastrointestinal lumenal concentrations. Where applicable, bone histology, urinary hydroxyproline measurements, bone biopsy and bone densitometry studies are obtained.

Major Findings: We have continued our studies of the effects of diphosphonates in a variety of metabolic bone diseases. Using Ca^{47} kinetics and balance techniques we have shown that diphosphonates can

decrease rates of bone turnover in a variety of patients and that ectopic calcifications in a variety of diseases, e.g., calcinosis and myositis ossificans progressiva, can be mobilized by the drug. Furthermore, in patients with idiopathic osteoporosis, diphosphonates can produce positive calcium balance. Bone densimetric measurements have shown no detectable decrease during the treatment period. To date, we have found no detectable increases in bone densitometry in patients on the drug.

We are also studying the mechanisms of calcium absorption in man. We are especially interested in defining the absorption mechanisms as a function of gastrointestinal lumenal calcium concentrations so that we can effectively diagnose and treat patients with calcium malabsorptions. Using the oral and intravenous Ca^{47} administration techniques together with computer deconvolution, we have been able to define a saturable curve for calcium absorption as a function of oral calcium load. There appears to be two components in this calcium absorption curve. One component saturates at a calcium load of about 200 mg whereas another component does not reach saturation at a calcium load of even 600 mg. The second component is probably due to diffusion. We are now studying patients with various calcium malabsorptions to define which component is altered by vitamin D or parathyroid hormone treatment. We are hoping that this technique will allow us to quantitate calcium absorption as a possible pathogenetic mechanism in metabolic and neoplastic bone disease.

Significance to Biomedical Research: The maintenance of calcium homeostasis is vital to man. Small changes in plasma calcium ion activity profoundly disturbs many physiological processes. The effects are related to the interaction of calcium ion in maintaining cell membrane integrity. Activation of adenyl cyclase, transport of substrates, as well as energy metabolism may all be related to intracellular or extracellular calcium concentrations. In cancer patients with bony metastases hypercalcemia may occur because bone is a major tissue component in the calcium homeostatic system. Our studies using pharmacologic agents which affect calcification may elucidate the mechanism of calcification and provide a therapeutic approach in treating bone metastases, metabolic bone diseases, and disorders of calcium homeostasis. In addition, our studies defining the calcium absorption mechanism may provide a therapeutic approach in treating patients with calcium malabsorption states and metabolic bone disease.

Proposed Course: We are emphasizing our studies of gastrointestinal calcium absorptive mechanisms as a function of lumenal calcium concentrations. We plan to study a variety of patients with calcium malabsorption states before and after treatment with vitamin D and parathyroid hormone.

Honors and Awards

None

Publications

Weiss, I.W., Fisher, L. and Phang, J.M.: Diphosphonate therapy in myositis ossificans progressiva. Ann. Int. Med. 74: 933-936, 1971.

Phang, J.M. and Weiss, I.W.: The maintenance of calcium homeostasis in man. Invited chapter in Handbook of Physiology. G. Aurbach (Ed.). In press.

Aurbach, G.D. and Phang, J.M.: Parathyroid hormone, calcitonin, and vitamin D. Invited chapter in Medical Physiology. V. Mountcastle (Ed.). In press.

Serial No. NCI-4014(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of the Immune Response in Normal and Pathological States

Previous Serial Number: SAME

Principal Investigator: Warren Strober, M.D.

Other Investigators: Thomas A. Waldmann, M.D., Z.M. Falchuk, M.D., Charles I. Heller, M.D., and J. Whitaker, M.D.

Cooperating Units: Digestive and Hereditary Diseases Branch, NIAMD; Medical Neurology Branch, NINDBS

Man Years:

Total: 5
Professional: 4
Other: 0

Project Description:

Objectives: Studies were directed toward determining the nature and mechanism of circulating and cellular immune responses to antigenic challenge in animals and humans, including patients with immune deficiency states, such as ataxia telangiectasia, agammaglobulinemia, intestinal lymphangiectasia and gluten-induced enteropathy. Insight into the cause of the high incidence of reticuloendothelial malignancies associated with these disorders was sought.

Methods Employed: Antibody responses in the individual immunoglobulin classes were measured using a modification of the C'1a fixation and transfer test of Borsos and Rapp. This test allows simultaneous quantitation of antibody in each of the immunoglobulin classes through the use of a quantitative C'1a fixation reaction. IgM antibodies are measured in a direct fixation reaction while IgG and IgA antibodies are measured in an indirect reaction using enhancing anti-antibodies specific for IgA and IgG.

The C'1a fixation and transfer test was used to measure antibody responses to arsanilic acid coupled with bovine serum albumin, fluid BSA and keyhole limpet hemocyanin. Local gastrointestinal immune responses were assayed by measuring mucosal immunoglobulin synthesis and mucosal antibody responses using solid phase immunoabsorbants. Techniques of organ culture were used to study gastrointestinal tissue in vitro.

In vivo antibody responses in patients with ataxia telangiectasia, Wiskott-Aldrich syndrome and other immune deficiency states were assessed by measuring antibody responses to Blood Group Substance, Vi antigen, Tularemia antigen and Pneumococcal Polysaccharide. In vivo delayed hypersensitivity was assessed with DNCB skin tests and homologous skin grafts.

Major Findings: An in vitro model of gluten-sensitive enteropathy (GSE) was developed in order to analyze the role of immunologic factors in this disease. Jejunal biopsy specimens were established in culture for as long as 48 hours. It was observed that alkaline phosphatase enzyme activity (a brush border enzyme) increased 1-2 fold in specimens from normal or diseased control individuals; in contrast, alkaline phosphatase activity in specimens from patients with GSE increased 4-5 fold, and entered or approached the normal range of values. Concomitant with this biochemical improvement, electron microscopic studies showed morphologic improvement: increase in number and length of microvilli. When specimens were cultured in the presence of gliaden, improvement in enzyme activity was reduced or absent in specimens from patients with active GSE, whereas gliaden had no effect on normal or control specimens. This inhibition in the recovery of enzyme activity by gliaden constitutes an in vitro model of gluten sensitive enteropathy.

The direct toxic potential of gliaden on jejunal mucosa was studied using the above in vitro model of GSE. Jejunal specimens obtained from patients in remission were cultured in the presence and absence of gliaden. It was found that enzyme activities rose in both circumstances. When the same group of patients were given a 7-10 day in vivo gluten challenge and specimens again obtained and cultured enzyme active rose in the absence of gliaden but not in the presence of gliaden. These findings indicated that the toxic effect of gliaden in vitro was only manifested after a period of in vivo gluten challenge, i.e., it was necessary to activate an endogenous mechanism (the immunologic process) before gliaden could exert an effect.

In broad ranging immunologic studies of GSE it was observed that patients make normal humoral and cellular immunologic responses to a variety of common antigenic stimuli. Using the C'1a fixation and transfer test it was shown that patients make normal IgA responses to blood group substances. In association with Dr. R. M. Blaese it was shown that peripheral lymphocytes from GSE patients undergo blastogenic responses to specific and non-specific mitogens. However, patients do not respond supra-normally to various preparations of gliaden.

The histocompatibility (HL-A) type of a large group of GSE patients was determined. 89% had HL-A8 in the second sub-locus. This contrasts with a 22-24% incidence of HL-A8 among normal individuals. The significance of this increased HL-A8 phenotype is unclear as yet. It may indicate that patients with GSE have an abnormal immune response gene (Ir gene) which allows them to mount immunologic responses to gluten within the GI mucosa. This is suggested by the fact that Ir genes in experimental animals are

linked to histocompatability genes.

Dermatitis herpetiformis (DH) is a skin disease associated with various immunologic abnormalities. Some two-thirds of patients with this condition have a gastrointestinal lesion identical to that seen in GSE. The HL-A type of DH patients was determined and it was found that 59% had HL-A8. Thus, this HL-A8 histocompatibility gene is found in both patients with GSE and DH and may be the genetic link between the two conditions.

In association with Dr. J. Whitaker of the NINDBS a family with hereditary sensory radicular neuropathy was intensively studied with regard to immunologic capabilities. Affected family members were found to have elevated serum IgA levels associated with normal serum IgM and IgG levels. The serum IgA was shown to be 7S in character. Metabolic turnover studies disclosed normal IgA survival characteristics. Studies of IgA responses using the C'1a fixation and transfer test showed no increased tendency to form IgA antibodies. Studies of gastrointestinal immunoglobulin synthesis showed increased gut IgA production with increases proportional to the increase in serum IgA. Thus, it appears most likely that the increased serum IgA results from increased gastrointestinal IgA synthesis. This finding is consistent with the view that patients with this condition have an occult gastrointestinal abnormality which may be of considerable significance in the pathogenesis of this disorder. These studies also suggest that 7S serum IgA elevations can result in lymphoid cells lining the gut.

Using the C'1a fixation and transfer test in rabbits, studies are continuing on the factors controlling IgA responses. Previous work has demonstrated that thymectomy greatly reduces rabbit IgA responses. More recently it has been shown that IgA responses are enhanced if antigen is given in Freund's adjuvant or if denatured antigen is used for immunization. This suggests that IgA responses are more dependent on T-lymphocytes than responses in other immunoglobulin classes.

Significance to Biomedical Research: Studies of patients with gluten-sensitive enteropathy have provided new insights into the operation of the local immune system. They suggest that local responses may be under the control of immune response genes or, alternatively may depend on the presence or absence of binding sites (receptors) which, in turn, are controlled by histocompatibility genes. The methods used to study patients with GSE hold promise of determining the precise role of immunologic factors in this condition. In addition, they can be applied to the study of immunologic processes in other gastrointestinal diseases.

Studies of the immune responses in patients with ataxia telangiectasia, and other immune deficiency states are leading to a greater understanding of the site and molecular nature of the defect in these various diseases. Patients with immune deficiency are more prone to develop neoplasma and it is therefore probable that immune processes constitute an important

defense against the development of such neoplasms. Studies of the immune defects encounted in immune deficiency diseases will provide a greater understanding of how immune processes prevent the development of neoplasms.

Proposed Course: Studies of the factors which control the IgA response will be continued. An attempt will be made to delineate the role of specific cellular systems in the IgA response. In this regard antigen associated with macrophages will be used to stimulate the antibody response in attempt to see whether macrophages play a necessary role in the development of IgA responses. In addition IgA responses in relation to carrier-dependent immune reactions will be studied in an attempt to show that IgA responses are dependent upon the normal functioning of a thymus-dependent lymphocyte population.

In humans, the C'1a fixation and transfer test will be used to study patterns of response in ataxia telangiectasia, and other immune deficiency states to a variety of antigens in order to relate these disorders to animal models of immunologic deficiency.

Patients with Wiskott-Aldrich syndrome, ataxia telangiectasia and other immune deficiency states will continue to be studied from a broad aspect. Attempts will be made to relate these immune deficiency states to animal models of immune deficiency such as thymectomized rodents, bursectomized birds and animals treated with various drugs or antiserums.

Continued studies of the role of immunologic factors in gluten-induced enteropathy and other gastrointestinal diseases will be carried out. In particular, an attempt will be made to show that locally produced anti-gluten antibodies are directly toxic to mucosal eipthelial cells in long term tissue culture. Patients with various forms of immune deficiency will be studied with their capacity to produce a local immunoglobulins. In this regard, an attempt will be made to study the pattern of intestinal immunoglobulin synthesis in a variety of dysgammaglobulinemias.

Honors and Awards

None

Publications

McFarlin, D.E., Strober, W. and Waldmann, T.A.: Ataxia-telangiectasia. Medicine. In press.

Strober, W., Blaese, R.M. and Waldmann, T.A.: Immunologic deficiency disease. Bulletin in Rheumatic Diseases. In press.

Serial No. NCI-4015(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of the Development and Function of Humoral and Cellular Mechanisms of Host Defense

Previous Serial Number: SAME

Principal Investigator: R. Michael Blaese, M.D.

Other Investigators: Thomas Waldmann, M.D., Warren Strober, M.D., J. J. Oppenheim, M.D., J. Wunderlich, M.D., J. Sever, M.D., R. Bruce, M.D., H. Hofstrand, M.D., and H. Kirchner, M.D.

Cooperating Units: Immunology Branch, NCI, and Laboratory of Microbiology, Immunology Section, NIDR, Infectious Diseases Branch, C. and F.R., NINDS

Man Years:

Total: 2
Professional: 2
Other: 0

Project Description:

Objectives: The objectives of this study were to determine mechanisms important to the development and function of both humoral and cellular immune response in animals and man; to determine the nature of the deficiency in immune function characterizing such disease states as the Wiskott-Aldrich syndrome, intestinal lymphangiectasia and advanced malignancy; and, to develop approaches to prophylaxis and/or therapy of the infectious and neoplastic disease frequently associated with defective immune processes. This work is directly related to Objective 2, Approach 1 of the National Cancer Plan.

Methods Employed: Antibody responses of experimental animals and man were determined after immunization with bacterial vaccines such as brucella abortus, S. typhosa, and pertussis; with particulate cellular antigens such as sheep, burro, and chicken RBC's; with purified protein antigens such as KLH, diphtheria and tetanus toxoids, guinea pig-γ-globulin and bovine γ-globulin; with purified polysaccharide antigens such as pneumococcal polysaccharides types I, II, III, and the V_i antigen of E. Coli. The antibodies were detected by standard techniques or by newly developed micro methods of bacterial agglutination, red cell

hemolysis, and passive hemagglutination using $CrCl_3$ as an agent to couple the antigens to a carrier RBC surface. In addition, modifications of the Farr antigen binding assay were used.

Cellular immune responses were measured by delayed hypersensitivity skin tests, contact sensitivity to DNCB, and skin allografts. Lymphocyte transformation in vitro was carried out utilizing non specific mitogens such as PHA, PWM and staph filtrate; specific antigens such as SLO, SKSD, diphtheria and tetanus toxoids, KLH and candida albicans; and allogeneic cells in mixed leukocyte culture. Lymphocyte function in vitro was also measured by production of mediators of cellular immunity such as lymphotoxin and MIF, and by cytotoxicity reactions with antibody sensitized target cells.

Studies of the characteristics of various cell surface receptors on immunocompetent lymphocytes and monocytes-macrophages utilized autoradiography, immunofluorescence microscopy, and cellular rosette formulation.

Major Findings: 1. Studies of factors controlling the development of immune competence were continued in neonatal rats and mice. In addition to the key role in this development played by the central lymphoid organs i.e., thymus, we have found a variety of other factors which are also important to the initiation of normal immune responsiveness. We have previously demonstrated that the ability of neonates to produce specific antibody following antigenic challenge develops in a distinct stepwise fashion and over a period of the first 4 weeks of life. This apparent sequential maturation of immune competence was found not to be dependent upon sequential differentiation of specific antigen reactive cells, but rather upon the maturation of components of the afferent limb of immunity. Macrophages play an essential role in the initiation of immune responses to many antigens, and it was demonstrated that immaturity of this system of cells accounted for much of the apparent immune deficiency in the neonate. Thus, when mature adult macrophages were given to the neonate, they produced antibody to a variety of antigens to which they were ordinarily unresponsive. It was also found that a variety of materials given to the neonates would result in accelerated maturation of their own macrophage population, and result in earlier immunocompetence. Thus, the adjuvant properties of such agents as endotoxin, BCG, alum, pertussis vaccine, silica, and poly A.U. were shown to function by macrophage activation.

It was shown that activation of the macrophage system of the neonates not only allowed "premature" antibody formation, but also was effective in preventing the development of specific immunologic tolerance to large doses of antigen given to newborns. Since several vertically transmitted oncogenic viruses are known to produce immunologic tolerance in the neonate, macrophage activation was utilized in an attempt to interfere with this tolerance induction, and subsequent tumor formation. It was found that C_3H mice treated at birth with Brucella vaccine, a potent macrophage activating substance, became resistent to mammary tumor

challenge as adults compared with untreated litter mates. Presumably immunologic tolerance to the MTV was interfered with by the macrophage activation procedure, and this resulted in non-specific immunoprophylaxis of the cancer induced by this vertically transmitted oncongenic virus.

2. Studies of the role of the control lymphoid organ responsible for humoral immunity in chickens, the Bursa of Fabricius, were continued. Agammaglobulinemia results when the bursa is removed surgically in the immediated post-hatching period, particularly if sublethal irradiation us used in association with surgery. We have previously shown that in vitro lymphocyte proliferation responses of these birds to non-specific mitogens, certain antigens, and allogeneic cells remains intact, while all humoral immune responses are deficient. It was found, however, that the in vitro lymphocyte transformation responses of these birds to some antigens, particularly sheep red blood cells and Brucella abortus organisms, was severely impaired in the agammaglobulinemic birds. In vitro cellular recombination experiments documented that the lymphocyte transformation responses to these antigens were dependent upon "B" lymphocytes, and also that a synergistic reaction between "B" and "T" lymphocytes was essential for normal immune responsiveness to many antigens.

Agammaglobulinemia also results when newly hatched chickens are given sublethal doses of cyclophosphamide, and a series of studies were carried out to characterize the immune competence of these animals. The cyclophosphamide induced immune defect of chickens appears to be entirely comparable to that following bursectomy and irradiation. In vitro lymphocyte transformation responses are intact to non-specific mitogens and allogeneic cells, while deficient responses to certain antigens were again demonstrated. Anti-immunoglobulin sera, while inducing a marked blastogenic response in normal chicken lymphocytes, was completely without effect on agammaglobulinemic lymphocytes, indicating that "B" lymphocytes are responding to this stimulant in vitro. Anti-thymocyte sera, on the other hand, induces identical proliferative responses in both normal and "B" cell deficient birds.

Autoradiographic studies of the lymphocytes combining with radio-labeled antigens were carried out in chickens to determine whether these lymphocytes carried immunoglobulin-like receptors on their surfaces. Immune and non-immune normal chickens contain lymphocytes capable of binding ^{125}I-KLH, and their binding can be inhibited by antisera to chicken immunoglobulin L-chains, suggesting that these cellular receptors contain immunoglobulins. Supporting this conclusion is data demonstrating that agammaglobulinemic "B" cell deficient chickens, do not have lymphocytes capable of binding radio-labeled antigens.

3. Studies of the immune deficiency diseases of man were continued. The Wiskott-Aldrich syndrome is an X-linked recessive disease characterized by thrombocytopenia, eczema, and multiple infections. We have extensively studied this disease over the past 5 years and have defined several severe

defects in cellular and humoral immune responsiveness in these patients. Among these defects, accelerated catabolism of all immunoglobulin classes and albumin was discovered associated with variably elevated rates of synthesis of these proteins. In fact, the hypercatabolic state for some proteins was completely masked by the increased protein synthesis. As an extreme case, we have now discovered 4 children with the syndrome that had clearly identifiable IgG paraproteins in their sera. Four additional patients with this disease studied at the Clinical Center have developed reticuloendothelial malignancy. The occurrence of these additional abnormalities associated with severe immune deficiency is strikingly similar to the malignancy and paraproteinemia occurring in SJL mice, perhaps providing an animal model for further investigation into relationship of reticuloendothelial malignancy and unregulated monoclonal immunoglobulin production.

Anergy and defective in vitro lymphocyte transformation are characteristic of many immune deficiency disorders and also several forms of malignant disease. It has been clearly shown that macrophages are essential to normal in vitro lymphocyte transformation responses to specific antigens and allogeneic cells, and a study was initiated to determine whether defective macrophage function could account for the anergy observed in several diseases. Lymphocyte suspensions free of monocytes and macrophages were obtained by glass bead column filtration of leukocyte rich plasma, and the degree of reconstitution of the blastogenic responses of these purified lymphocytes by macrophages obtained from anergic patients was determined. The antigen induced responses of column purified (macrophage depleted) lymphocytes were decreased by over 90% and it was found that readding 1 macrophage to 10 lymphocytes was optimal for the complete reconstitution of the lymphocytes blastogenic response. Macrophages from anergic subjects were generally just as effective in reconstituting the responsiveness of normal purified lymphocytes as were normal macrophages. By contrast normal macrophages were unable to reconstitute or promote responses by lymphocytes from anergic patients, demonstrating that the defective in vitro lymphocyte responses of anergic patients could not be explained by defective macrophage function in vitro.

Subacute sclerosing panencephalitis is a universally fatal chronic encephalitis caused by rubeola virus. In association with Dr. John Sever of the NINDS, we have evaluated the immunologic status of 9 patients with this disease and have instituted a therapy protocol utilizing "transfer factor." Immunologically, these patients are characterized by generally intact humoral and cellular immune systems until late in the disease when anergy tends to supervine. It has been postulated that the basic defect in the patient leading to susceptibility to this disorder is a specific deficiency in cellular immunity to the measles antigen, allowing the virus to persist in a latent state until full encephalitis develops 4-8 years after initial infection. Because of the observation of Lawrence that a soluble extract of human lymphocytes (transfer factor) is able to transfer specific cellular immunity to non-immune individuals,

"transfer factor" from measles immune donors was prepared and given periodically to the SSPE patients. Preliminary results of this therapeutic trial indicate that in 6 of 7 patients, progression of neurologic disease has apparently halted, and in 2 of these patients, improvement in neurological states has occurred. Treatment with transfer factor will be continued for at least 6 months, and then final immune and neurological evaluation will be completed.

Significance to Biomedical Research: The present studies extend our understanding of the diverse, yet interrelated mechanisms contributing to the development and normal functioning of immune system in animals and man. They indicate that disorders of immune function may be related to the absence or malfunction of such central lymphoid organs as the thymus or Bursa of Fabricius or to immaturity of such axillary systems as the macrophage required for the processing of antigens in the initiation of an immune response. They also indicate that the macrophage plays multiple roles in a normal immune response, being both important in the initiation or afferent limb of such responses and also as the ultimate effector cell in many immune reactions. Our studies on macrophage activation may provide an explanation for the mechanism by which such agents as BCG have shown some therapeutic effectiveness in the treatment of cancer. That is, BCG can activate macrophages for more effective recognition of antigens and thereby promote a more efficient immune response BCG will also activate the efferent limb killing function of the macrophage to allow a more efficient destruction of the malignant cell. In addition, the ability to manipulate the degree of activation of macrophages in the neonate has provided us with a mechanism of non-specific prophylaxis of malignant disease caused by vertically transmitted tumor viruses which ordinarily induce tolerance when exposed to the neonatal animal. Our studies of lymphocyte function in patients with immune deficiency and advanced malignancy have extended our understanding of the nature of the reactions produced by different classes of lymphocytes and the observation that some of these functions may remain intact in severely anergic individuals may provide us with a mechanism of providing effective immunotherapy for malignancy occurring even in severely immune deficient patients.

Proposed Course: Continuing studies directed toward understanding the normal pathways involved in the development and function of the immune system and application of this new knowledge in approaches to the prevention of therapy of malignant disease.

Honors and Awards

None

Publications

Weiden, P.L. and Blaese, R.M.: Hemolytic anemia in agammaglobulinemic chickens: A model of "autoimmune" disease in immune deficiency. J. Immunol. In press.

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Weiden, P.L. and Blaese, R.M.: Hereditary thrombocytopenia: Relation to Wiskott-Aldrich syndrome with special reference to splenectomy. J. Pediat. 80: 226-234, 1972.

Weiden, P.L., Blaese, R.M., Strober, W., Block, J.M., and Waldmann, T.A.: Impaired lymphocyte transformation in intestinal lymphangiectasia: Evidence for at least two functionally distinct lymphocyte populations in man. J. Clin. Invest. In press.

Blaese, R.M., Oppenheim, J.J., Seger, R.C., and Waldmann, T.A.: Lymphocyte-macrophage interaction in antigen induced in vitro lymphocyte transformation in patients with the Wiskott-Aldrich syndrome and other diseases with anergy. Cell. Immun. In press.

Jacobs, R., Blaese, R.M. and Oppenheim, J.J.: Inhibition of antigen stimulated in vitro proliferation of thymic dependent chicken spleen cells by specific activity. J. Immun. In press.

Kirchner, H., Blaese, R.M., Hofstrand, H. and Oppenheim, J.J.: Defective in vitro spleen cell proliferative responses to antigen in agammaglobulinemic chiakens. J. Immun. In press.

Waldmann, T.A., Strober, W. and Blaese, R.M.: Metabolism of immunoglobulins In Progress in Immunology. Pages 891-903, 1972.

SUMMARY STATEMENT
Laboratory of Pathology
General Laboratories and Clinics
National Cancer Institute
July 1, 1971 to June 30, 1972

Introduction

The diagnostic and research activities of the staff of the Laboratory of Pathology are listed by sections. The research accomplishments are especially impressive when one considers that the majority of the staff participate to some extent in the diagnostic services for Clinical Center patients. It is by offering opportunities and facilities for laboratory research that we have been able to recruit and retain an outstanding group of anatomical pathologists who have been able to give vital support to the clinical programs of NCI as well as all of the other Institutes with patients in the Clinical Center.

The research programs of the laboratory have been involved with:

A. Immunopathology
B. Viral Oncology and Cell Biology
C. Comparative Pathology and Carcinogenesis

Some of the more significant research accomplishments in which staff of Laboratory of Pathology participated (either as principal investigators or collaborators) include:

A. Immunopathology:

1. Induction of malignant lymphomas in mice under prolonged immunosuppression and persistent antigenic stimulation. These experiments provide model systems analagous to induction of lymphomas in humans with organ transplants.

2. Ability of antilymphocyte serum and heterologous serum to reduce interferon production. These findings may also relate to the increased incidence of lymphomas in humans with organ transplants.

3. Electron microscopic localization of immunoglobulin E on the surface membrane of human basophils.

B. Viral Oncology and Cell Biology:

1. Finding of elevated EB virus antibody titers in patients with Hodgkin's disease of the lymphocyte depleted type. It was not until the series of Hodgkin's disease patients were subdivided into histological types that the relationship of EB antibody titers to the lymphocyte depleted type became evident.

2. Conclusive evidence of the epithelial origin of polyoma-virus induced salivary gland tumors by utilization of chromosome markers.

3. Demonstration of ability of cyclic AMP to restore normal morphological characteristics to certain sarcoma cells and to alter cell motility.

4. Demonstration of chimeric nature of lymphoma cell line induced by Herpesvirus saimiri from marmoset indicating that the cell line could not be of monoclonal origin.

5. Evidence that cyclohexamide increases activity of interferon inducers in human cells which may provide a means for producing large amounts of human interferon for antiviral chemotherapy.

6. Effect of Rifampicin in inhibiting poxvirus morphogenesis.

C. Comparative Pathology and Carcinogenesis:

1. Finding that fiber size and durability are key factors in mesothelioma induction by asbestos.

2. Induction of epidermoid carcinoma in lungs of rats by direct implantation of cigarette smoke condensate pellets.

Many members of the staff, in addition to their laboratory and service work, serve on numerous national and international committees, editorial boards, etc. Some of the principal activities of this sort are: Dr. Stanton, Scientific Editor of the Journal of the National Cancer Institute; Dr. Dawe, Associate Editor for International Journal of Cancer and Cancer Research; Dr. Rabson, Associate Editor of JNCI and Cancer Research; Dr. Grimley, Associate Editor of JNCI; Dr. Friedman, Associate Editor of Journal of Virology; Dr. Berard, Pathology Editor, Journal of Reticuloendothelial Society; Dr. O'Conor, Chairman of U.S.A. National Committee for UICC and Chairman of Commission on Epidemiology, UICC; Dr. O'Conor and Dr. Thomas, WHO Collaborating Centers for classifications of neoplasms; Dr. Dawe, Chairman of Committee on Comparative Oncology, UICC; Dr. Rabson served as an Ad Hoc member of the Pathology Study Section (DRG) in January 1972. Other committees, symposia, etc. participated in by the staff are listed in the annual reports.

Dr. Theodore Pincus resigned in June 1971 and his position was filled by Dr. Donald E. Henson on July 1, 1971.

Drs. Takayama, Yun and Hirano completed their research fellowships during 1971 and returned to their home countries. Dr. Phillip Periman, Dr. Nubia Munoz and Mr. Bert Zwiers spent part of the year in Dr. Rabson's laboratory and Dr. Teruyo Sakakura worked as a Research Associate with Dr. Dawe. Dr. Richard A. Malmgren retired on April 30, 1972. Dr. Roger O'Gara died of myocardial infarction on January 25, 1971.

The staff of the Laboratory of Pathology were either senior authors or co-authors of 67 papers and 9 abstracts published in 1971. There are 10 publications to date (April) in 1972.

Surgical Pathology Section:

Dr. O'Conor and Dr. Powell, together with other pathologists participating in the diagnostic services of the Laboratory of Pathology, are responsible for the diagnostic reports in Surgical Pathology. Approximately 6,229 specimens were accessioned. In addition there were approximately 3,000 bone marrows sectioned. Dr. O'Conor and his staff regularly make rounds with Dr. Ketcham and the staff in the Surgery Branch of the NCI and show most of the surgical biopsies and operative specimens at the weekly Surgical Pathology Conference.

Postmortem Section:

Dr. Thomas, the residents and other staff were responsible for 248 autopsy examinations. A gross review is held with the residents and clinical staff each morning at 8:30 a.m. and the staff and residents present selected autopsies at numerous clinical-pathologic conferences. The secretaries continue to call many scientists in various laboratories at NIH before an autopsy is performed so that fresh, unfixed human tissues can be used for research studies.

Use of a new autopsy permission form for the Clinical Center started in January, 1972. It gives permission to enucleate the eyes which then can be used for pathology studies or in some instances for other purposes, such as corneal transplantation or physiological or biochemical studies. This new program was worked out with Dr. Carl Kupfer and Dr. Toichiro Kuwabara of the National Eye Institute and has been very successful. Since its inception approximately 31 pairs of eyes have been removed by the staff in the Laboratory of Pathology and furnished to the staff of the NEI. The permission rate to remove eyes at postmortem is more than 95%. These constitute an important additional pathological study of the patients dying at the Clinical Center and are an invaluable source of corneas needed for transplantation.

Publications of studies on human material: A number of studies on human disease were published by the residents and staff of the Laboratory of Pathology during 1971 and other studies are now going on which will lead to publication. These include Gilbert's disease (Barth, Grimley - 1), electron probe analysis of iodine in the human thyroid (Banfield, Grimley - 3), Hodgkin's disease (Berard, Thomas, O'Conor, Correa - 4, 18, 49, 50, 56), carcinoma of the cervix (Thomas - 16, 53), hepatocellular carcinoma (O'Conor - 22), Burkitt's tumor (Berard - 26), pulmonary alveolar hemorrhage (Buja - 31), iron in the myocardium (Buja - 35), multiple sweat gland carcinomas (Krueger - 36), lymphoreticular tissue in Steinbrinck-Chediak-Higashi syndrome (Krueger, Grimley - 39), graft vs. host reaction (Krueger, Berard - 40), toxoplasmosis (Roth - 48), cancer of larynx and pharynx (Roth - 52), culture of leukocytes from patients with cystinosis (Berezesky, Grimley - 57), malignant melanomas (Malmgren - 58), human cytomegalovirus (Rabson - 59), immunoglobulin E on surface membrane of human basophils (Grimley - 60), and coffee drinking and bladder cancer (Dunham - 67). (Reference nos. are to 1971 publications list).

Other research activities by staff in Surgical Pathology and Postmortem Sections: In addition to his diagnostic pathology services, Dr. O'Conor conducts research in electron microscopy and in epidemiology. Included are studies on

human and Simian herpes virus (in collaboration with Dr. Rabson and Dr. Kirschstein), studies by EM of selective human neoplasms including liver cancer and penile cancer in Ugandan patients. Epidemiological studies include a review and subclassification of Hodgkin's disease in Connecticut in order to establish age-specific incidence rates and a review of cancer of the oral cavity and respiratory tract in Uganda.

Dr. Powell, in addition to his diagnostic pathology responsibilities, is collaborating in a number of human and animal studies including sexual maturation in olfactomized female rats, bronchogenic carcinoma, maturation of ovarian follicles in women with decreased production of gonadotrophic hormones and pulmonary aspergillosis.

A complete list of other human and experimental studies currently conducted by the resident staff is given in the individual annual reports.

Laboratory services for human tissues: Miss Bowling and her staff continued to provide excellent histological slides and special stains for the human tissues. She also provided valuable consultation on special staining and preparation of human tissue to many clinical associates and other NIH scientific staff. More than 51,000 slides were made on the surgical pathology and postmortem tissues.

Photography for human material was provided by Mr. Kenzie Edwards and by Mr. James Banks who, in addition, maintains the slide files which now contain more than a million slides. Access to these and to the filed gross and microscopic photographs is invaluable for the increasing number of clinico-pathologic studies.

After several years of development and pilot studies, the pathology data processing system developed by Drs. Thomas and Pratt has been placed in operation. In brief, this involves storing pathology diagnoses using a terminal and the Wylbur text-editing program and then end-coding these diagnoses by computer. Surgical pathology diagnoses for 1970 are completed and the 1971 diagnoses are partially processed. It is anticipated that this system will result in an up-to-date, current processing of all pathology diagnoses when it is fully developed. Dr. Donald Henson is now working in this program.

Cytopathology Section:

The cytopathology Section staff is responsible for diagnostic reports on all exfoliative cytology specimens: sputum, urine, cervical-vaginal smears, CNS fluid, effusions, etc. In addition, Dr. Chu and the staff examine cellular materials for karyotyping and chromosomal abnormalities. Approximately 5,132 specimens (13,984 smears) and approximately 191 chromosomal analysis specimens (1,910 smears) were examined during 1971. Studies published included cytology and immunologic factors in malignant tumors (Malmgren - 7, 43, 44, 58). Also Dr. Malmgren initiated a contract with the AEC group at Los Alamos to study the automation of cytology. The details of this are given in the contract report. Dr. Chester Herman will become Project Officer of this project after Dr. Malmgren retires.

Ultrastructural Pathology Section:

Dr. Grimley and Dr. Henson and other participating staff members are responsible for examination of human tissues, teaching of residents and independent research. The research projects include studies on the role of membranes in arbovirus replication, the effects of rifamycin derivatives on the structure and morphogenesis of poxviruses (Grimley - 27, 28, 41), evaluation of the significance of tubuloreticular structures in neoplastic cells, virus-infected cells and cells of patients with connective tissue diseases, and an examination of the effects of viral transformation (SV40) of human thyroid medullary carcinoma cells (Wivel, Grimley - 1972). Dr. Henson published studies on mumps virus sialoadenitis (54) and myocarditis and pneumonitis with type 21 adenovirus infection (55).

Hematopathology Section:

Dr. Berard, Dr. Krueger and the residents and other staff participants are responsible for a variety of special studies of patients with leukemia, malignant lymphomas and immunoproliferative diseases. A number of publications dealing with these human diseases have been cited in an earlier section of this report. Experimental studies published in 1971 included production of malignant lymphomas in mice by immunosuppression and antigenic stimulation (Krueger, Berard - 9; Krueger - 30, 45, 46), graft-versus-host disease (Krueger, Berard - 13). These studies have been continued and a number of additional publications are in press or have been published in 1972.

Dr. Elaine Jaffe completes residency training in June 1972 and will then work with Dr. Berard as a Staff Fellow. They have started a number of clinicopathologic studies on reticulum cell sarcoma, Wiskott-Aldrich syndrome, vascular invasion in Hodgkin's disease and a review of the extramedullary manifestations of chronic myelogenous leukemia.

Viral Oncology and Molecular Pathology Section:

Dr. Rabson, Dr. Friedman and Dr. Levin have continued their studies on a number of problems related to oncogenic viruses, interferon and the production of viral RNA. Publications in 1971 resulting from these studies (Periman - 3), (Friedman - 11, 14, 21, 24, 29, 37, 65, 66), (Levin, Friedman - 12), (Rabson, O'Conor - 15), (Berezesky, Grimley, Tyrrell, Rabson - 25), (Rabson - 47) are included in the bibliography of the laboratory for 1971.

Dr. Friedman and his associates have continued to make progress in the study of replication of arboviruses and the mechanisms of action of interferon. They have utilized polyacrylamide gel electrophoresis to further analyze arbovirus RNA forms and have carried out extensive studies on induction and superinduction of interferon. Increased yields of human interferon have been found after treatment of human cells with interferon inducers plus cyclohexamide. In collaboration with Dr. Ira Pastan's group, they have studied effects of cyclic AMP on growth of sarcoma cells in vitro and shown that the cyclic AMP restores some properties of normal fibroblasts to the sarcoma cells. They have also shown that the cell fusion factor of murine leukemia viruses can be demonstrated in viral concentrates and have studied the mechanisms of fusion

process. Since July 1971, Dr. Friedman has been a Visiting Scientist at the National Institute for Medical Research in Mill Hill, London, England. While there, he has developed a cell free system for producing viral peptides and is using this system to analyze the precise localization of the effect of interferon on translation of viral messenger RNA.

Dr. Rabson and Dr. O'Conor have continued their studies of oncogenic herpesviruses with emphasis on Herpesvirus saimiri. They have isolated a lymphoid cell line from a lymphoma of the marmoset induced by H. saimiri. The cells of this line carry H. saimiri in a latent or repressed form and can be induced to produce infectious virus on contact with a susceptible "indicator cell". The frequency of cells yielding virus can be increased by treatment with BUDR and argenine-deficient medium. With Dr. Periman, they have fused the marmoset lymphoma cells with mouse cells using ultraviolet-irradiated Sendai virus and produced hybrid cells. These hybrids still contain H. saimiri, however, there is an inhibition of viral induction in the hybrids. In collaboration with Dr. Chu, they have shown that the marmoset lymphoid cell line contains both male and female cells thus indicating that the line cannot be of monoclonal origin as has been suggested for many human lymphoid cell lines on the basis of isozyme studies. Further studies of mechanisms of latency and induction in lymphoid cells carrying herpesviruses are in progress.

Dr. Friedman will return to his laboratory in October 1972 and at that time he, Dr. Levin and their technical staff will be transferred to Dr. Ira Pastan's laboratory.

Dr. Nubia Munoz will start as a Visiting Associate with Dr. Rabson in October 1972. Dr. Phillip Periman is continuing to work part-time in Dr. Rabson's laboratory.

Comparative Oncology Section:

Drs. Dawe, Banfield, Snell, Stanton, Dunham and Mulay are the professional staff members of this section. Dr. Sakakura is a Visiting Associate and works in Dr. Dawe's laboratory.

The experimental studies include: (1) morphologic characterization of neoplasms in a wide variety of laboratory and wild animals, rodents, primates, lower vertebrates and invertebrates; (2) mechanisms involved in tumor development; and (3) chemical carcinogenesis. Publications included studies of the epithelial origin of polyoma induced salivary gland neoplasms (Dawe - 5), carcinogenicity of plant extracts from Curacao (O'Gara - 20), procarbazine induction of leukemia in primates (O'Gara - 23), azo-dye carcinogenesis (Mulay - 38), EM studies of Shope fibroma (Banfield - 42), histidine decarboxylase and other enzymes in gastric carcinoids (Snell - 51), effects of nitrosamine derivatives on the crayfish (Harshbarger, Stanton - 64), and mechanisms of mesothelioma induction with asbestos and fibrous glass (Stanton, Wrench - 1972).

Mr. Ralph Isenburg provided photographic services for the experimental animal studies and photomicrography for publications by the staff of the Laboratory of Pathology. He, together with the technical staff from Dr. Grimley's and

and Dr. O'Conor's EM laboratories, developed plans to consolidate the dark room facilities in Building 8 with his photography laboratory in Building 10.

Pathological Technology Section:

Miss Betty Sanders and her staff continued to provide superior service to members of the Laboratory of Pathology staff and to approximately 100 scientists in other laboratories of the NCI. She has successfully supervised two contracts; one to produce routine H&E stained sections of animal tissues and the other to store paraffin blocks. In addition she and her staff continued to provide excellent histological slides in spite of moving into smaller space in Building 8 and while preparing plans to move the laboratory to Building 37 sometime in 1972.

LABORATORY OF PATHOLOGY

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Serial No. NCI 500
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Histogenesis and Pathology of Induced and Spontaneous Tumors of Laboratory Animals

Previous Serial Number: Same

Principal Investigator: Katharine C. Snell, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional:
Other:

Project Description:

A. Praomys (Mastomys) natalensis: (Mastomys)

Objectives: (1) To study and describe the tumors and other lesions that develop spontaneously, some of which appear to be counterparts of human diseases. (2) To ascertain the mechanism of the effect on host animals bearing transplantable carcinoids that originated within the glandular stomach. (3) To study the enterochromaffin and enterochromaffin-like cells of the gastrointestinal tract of normal Mastomys and of those that have primary gastric carcinoids.

Methods Employed: (1) Most Mastomys are allowed to live as long as possible in order to furnish information on tumor incidence. Prior to necropsy, blood serum from each animal is collected and frozen. This will be used to study the immunoglobulins in animals with pyelonephritis, the relationship of autoantibodies to striated muscle in animals with thymomas, and the histamine content in animals with carcinoids of the glandular stomach. With Dr. Carel Hollander of Rijswijk, The Netherlands, a paper has been written and will shortly be submitted for publication. This paper combines the findings in the colony in Rijswijk and the one at NIH and describes tumors and various other lesions of the testis and seminal vesicles.

(2) Transplanted carcinoid tumor tissue and blood serum from Mastomys bearing transplants of argyrophilic carcinoids have been shipped to Dr. Syun Hosoda of Nagoya, Japan. Dr. Hosoda is continuing to work on the determination of histamine, histidine decarboxylase and other enzymes present in this tissue.

(3) For collaborative experiments, Mastomys have been sent to Dr. Rolf Håkanson of Lund, Sweden and also to Dr. Enrico Solcia of Pavia, Italy. Dr. Håkanson is studying the carcinoids to determine whether they originate from enterochromaffin-like cells, and Dr. Solcia is interested in the same problem, but is using slightly different methods. Both investigators are doing further electron microscopy on the tumors and on normal gastric mucosa.

Major Findings: (1) Malignant argyrophilic carcinoids occur in approximately 1/2 of Mastomys 18 months of age or older. Thymomas and thymic hyperplasia appear in a high percentage of animals, particularly in females, and are often associated with myositis and myocarditis. In a total of 391 Mastomys over 18 months of age, we are reporting 9 adenocarcinomas of the rete testis, 6 seminomas, and 12 carcinosarcomas of the seminal vesicles.

(2) Mastomys bearing transplanted argyrophilic carcinoids develop multiple ulcers of the stomach and duodenum. Pathologic changes similar to those of human malabsorption syndrome are seen in the jejunum and ileum. The transplanted tumors do not contain or produce measurable amounts of gastrin. They do contain appreciable amounts of histamine, specific histidine decarboxylase and L-amino acid decarboxylase, which may be related to the inherent properties of the parent cells, probably histamine-containing argyrophil cells, distributed in the gastric mucosa.

(3) Dr. Håkanson has found that the endocrine cells in the oxyntic gland area of the glandular stomach of Mastomys can be identified by silver staining and by fluorescence microscopy after treatment with L-dopa. From electron microscopy there appear to be at least 3 different endocrine cells in the oxyntic gland area. In the oxyntic gland area of non-tumor-bearing Mastomys the activity of histidine decarboxylase is very low, but in the tumors, the enzyme activity is uniformly high. The tumors contain not only histidine decarboxylase but also dopa decarboxylase. Vitamin B binding proteins occur in the oxyntic gland area. Tumor tissue seems to be devoid of such binders. Dr. Solcia also reports that the best candidate for the origin of the carcinoids is the enterochromaffin-like cells, but that the ultrastructural pattern of tumor cell granules differs from those of Mastomys' normal ECL cells. Besides tumors, Dr. Solcia found a diffuse hyperplasia of endocrine cells displaying all staining and ultrastructural features of ECL cells.

Significance: Mastomys are becoming of increasing importance as experimental animals, since in many ways their diseases parallel those of man (gastric carcinoids, osteoarthritis, glomerulonephritis, multiple endocrine adenomas, for example). These animals may be useful for determination of the chronologic and functional relationships between thymoma and anti-striated muscle antibodies, and possibly polymyositis and myasthenia gravis. It seems likely that study of their gastric carcinoids may shed light upon the relationship between various types of enterochromaffin cells, pluriglandular adenomas and the production of histamine. The continued study of untreated Mastomys is important in order to establish a baseline for future experimental work.

Proposed Course: Collaborative studies will be continued. Old Mastomys now on hand will be necropsied and histologic examination of their tissues made. Hopefully a monograph on the tumors and other lesions will be written within the next year or so. Because of space restriction, we do not intend to continue the study of aging Mastomys beyond those animals now on hand.

B. Study of the Offspring of Pregnant Rats Treated with Diethylstilbesterol

Objectives: To see whether it is possible to induce tumors in the offspring of rats by treatment of the mothers when pregnant and thereby shed more light on the recent work of Herbst et al (New England J. Med. 284: No. 16, 1971) and of Greenwald et al (New England J. Med. 285: No. 7, 1971). These authors found that the rare tumor, adenocarcinoma of the vagina, was appearing in a large number of young women whose mothers were treated with diethylstilbesterol during pregnancy for threatened abortion.

Methods Employed: Female F344 and M520 rats will be treated with doses of diethylstilbesterol approximately equivalent to those given the pregnant women, beginning about day 12 of pregnancy. It is planned to put aside the offspring of these rats and to watch both the females and males for the development of any type of tumor.

Major Findings: No results as yet. However, in an effort to determine the proper dose of diethylstilbesterol, we have learned that when double the human equivalent dose is given to pregnant rats a day or two after implantation, all fetuses are resorbed or aborted.

Significance: If tumors are induced in offspring of our treated rats, it will help substantiate the inferred relationship in the human patients. If tumors or other abnormalities appear in the male offspring, it should stimulate a study of the young men whose mothers received diethylstilbesterol during pregnancy. No report on this subject has yet appeared in the literature.

Proposed Course: The experiment has just begun.

C. Untreated Rats

Objectives: To determine the incidence of tumors and other lesions that occur spontaneously in the inbred Sprague-Dawley strain of rats. This is a relatively new strain developed by Dr. Carl Hansen, Laboratory Aids Branch. It is being used by several investigators but no pathologic study has been made on the animals.

Methods Employed: Weanling inbred Sprague-Dawley rats are being set aside for aging and groups of both males and females will be killed at 3-month intervals, beginning when the animals are 12 months old. The longevity of the rats will determine the oldest age group to be studied, but this will probably be from 27-30 months.

Major Findings: None so far.

Significance: A careful pathologic study will be useful to any investigator using these rats for experimental purposes because it is important to know the spontaneous incidence of tumors and other lesions. Also the knowledge gained from a study of neoplasms and other lesions that occur in laboratory animals can provide a basis and possible analogy to the study of the tumors and other lesions that occur in human beings.

Proposed Course: If space for aging large numbers of rats becomes available, we propose to study other recently developed inbred strains.

D. Mechanism of Fetal Resorption Following Treatment of Pregnant Rats with the Polypeptide, Nisin, or Some of its Fractions

Objectives: To study the changes that take place in the fetus and in the uterus and other organs of pregnant rats treated with nisin or its fractions. This is a collaborative study with Dr. Erhard Gross of NICHD.

Methods Employed: Dr. Gross treats pregnant rats with various doses of the compound under study, by various routes, and on various days of pregnancy. We perform the necropsies with him and examine the histologic sections.

Major Findings: Nisin or its fractions appears, under certain circumstances, to cause partial or complete fetal resorption. It is too early to conjecture about the mechanism. The fractions, however, especially when given intravenously, causes severe damage to the kidneys as evidenced by precipitation of the compound within the renal tubules.

Significance: The ultimate aim of the group in NICHD is probably the discovery of various drugs that can be used in human population control. If so, it is highly desirable that they be aware of the potential hazards as well as the mechanism of action.

Proposed Course: Continuation of necropsies and examination of histologic sections.

D. Monkeys

Objectives: Originally the objective of this experiment was to determine whether 3-methylcholanthrene injected into the wall of the stomach would induce gastric carcinoma. Since, after many years of observation, no gastric cancers have been found, we are keeping the remaining monkeys to study any tumors or other lesions that may occur with age.

Methods Employed: 3-methylcholanthrene was injected or implanted in the wall of the stomach of rhesus monkeys 18 to 19 years ago. The monkeys, which are now 23 or more years old are being observed and allowed to live as long as possible. Prior to necropsy, skeletal surveys will be made by Dr. Doppman of the Clinical Center X-ray department. Also, brains will be perfused by Dr. Henryk Wisniewski of the Albert Einstein College of Medicine, who is interested in studying the aging process of neurons by electron microscopy.

Major Findings: Nothing significant has been observed in the stomach of any monkey necropsied. Three cases of spinal ankylosis, bilateral carcinoma of the kidney, diabetes with hyalinization of the islets of Langerhans, and adenocarcinoma of the ascending colon are the principal pathologic findings so far observed.

Significance: With the increasing use of monkeys in cancer research it is important to study old animals to determine the incidence of spontaneous disease. Very few colonies of monkeys of known and advanced age are in existence so that our animals constitute an important group for future study. Hyperostotic spondylosis is a disorder that is currently the subject of considerable clinical study. These monkeys may offer an opportunity for observing changes in bone which are almost never available in human autopsy material. The finding of carcinomas of the kidney and of the colon as well as the hyalinized islets in the diabetic monkey suggest that monkeys, if allowed to live long enough, may indeed develop many of the same diseases found in man.

Proposed Course: Continued observation of monkeys with necropsies as necessary with collaborative studies as indicated under Methods.

E. Transplantation of Tumors

Objectives: To carry interesting or unusual tumors in transplant for possible future study in this laboratory or by other investigators.

Methods Employed and Major Findings: The following tumors are carried by subcutaneous, intraperitoneal, or intramuscular injection:

494..Adrenal cortical carcinoma, OM/N rat. Tumor has been used by several investigators for hormone research and is currently being studied by Dr. Max Sweat of the University of Utah, and by Dr. Evan Cameron of the Welsh National School of Medicine, Cardiff, Wales, and in tissue culture by Dr. L.M. Edelstein of the New England Medical Center, Boston.

555..Gastric adenocarcinoma induced by 2,7-FAA in ACI/N rat. Tumor develops metaplastic bone, bone marrow and cartilage in center.

328..Gastric adenocarcinoma, C57BL mouse, induced by intramural injection of 3-MCA into the wall of the stomach. Mice in which this tumor has grown for a long time develop hypervolemia. Currently being studied is tissue culture by Dr. L.M. Edelstein of New England Medical Center, Boston.

586..Skin appendage tumor induced in ACI/N rat by oral administration of 2,7-FAA, being studied by Dr. Shu Wen How and in tissue culture by Dr. L.M. Edelstein of the New England Medical Center, Boston.

673,684,691..Malignant argyrophilic carcinoids that originated in the glandular stomach of untreated Mastomys.

648..Hepatic cell carcinoma that originated in a Buffalo rat that received aflatoxin in alcohol intragastrically by stomach tube.

670..Mesothelioma that originated in the peritoneal cavity of a Mastomys.

Significance: The study of transplantable tumors continues to be a useful tool for investigators interested in hormone research, tissue culture, biochemistry, and transformation of tumor type during successive transplant generations.

Proposed Course: To maintain the tumors in transplant and make them available to other investigators.

Publications:

1. Hosoda, S., Nakamura, W., Snell, K.C., and Stewart, H.L.: Histidine decarboxylase in the transplantable argyrophilic gastric carcinoid of Praomys (Mastomys) natalensis. Biochem. Pharmacol. 20: 2671-2676, 1971.

2. Hollander, C., and Snell, K.C.: Tumors of the adrenal gland in rats. In Turusov, V. (Ed.): Manual on the Pathology of Tumours in Laboratory Animals. (in press).

Serial No. NCI 501
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Carcinogenesis in the Cheek Pouch of Hamsters or in the Esophagus of Hamsters and Rats: A Search for Factors in the Environment that may be Related to Cancers in Man

Previous Serial Number: Same

Principal Investigator: Lucia J. Dunham, M.D.

Other Investigators: Roger W. O'Gara, M.D. (Deceased)

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional:
Other:

Project Description:

Objectives: To identify substances or factors in the human environment that induce or influence the development of cancers.

Methods Employed: 1) The effects of several plants used for herb teas (Annona, Heliotropum, Krameria, Acacia) and under consideration as possible causes of cancers of the esophagus in Curacao, NWI, are tested in the hamster cheek pouch or esophagus with and without added injury to tissues by calcium hydroxide (lime).

2) The effects of arecoline, an alkaloid present in the betel quid used in Southeast Asia, is tested in hamsters by oral administration.

3) The abdominal portion of the esophagus of hamsters or rats has been chronically constricted by means of surgically placed cuffs of polyethylene tubing, and carcinogenesis is attempted by means of intra-esophageal instillation of benzpyrene.

4) Dunham serves as a consultant in connection with the current posthumous studies of plant materials conducted in the laboratory of Dr. O'Gara. She will correlate the results, and will prepare an article for publication.

Major Findings: 1) In an earlier experiment, Annona muricata plant produced advanced atypia in a cheek pouch and an esophagus of hamsters, and Heliotropum ternatum apparently produced an esophageal papilloma. A current experiment, nearly completed, involves intensive treatment of upper

gastrointestinal sites with Annona and Heliotropum, and also with Krameria and Acacia which have produced sarcomas at subcutaneous sites in rats. In the new experiments Annona has apparently produced a gastric papilloma. Though Acacia brew administered subcutaneously to rats is strongly sarcogenic (O'Gara: unpublished observations), highly concentrated Acacia brew administered by mouth to hamsters has so far not been carcinogenic.

2) The extended experiments with arecoline have not resulted in the production of additional papillomas of esophagus, though this lesion was found in an earlier test of arecoline.

3) An experimental group of hamsters with restricting cuffs at esophagus and subjected to up to 10 instillations of benzpyrene in dimethyl sulfoxide is under observation. Adjacent fibrosis has followed insertion of the cuffs. However, extensive atypia of esophageal mucosa or carcinoma have not so far resulted in hamsters with cuffs subjected to treatment with benzpyrene.

Significance: The frequencies of cancer in the human mouth and esophagus vary widely by geographic area, and the ratios by sex vary also. Constrictions, dilatation, esophagitis, and scarring are among precursor lesions of cancer of the esophagus that have been reported. Environmental factors may be important in the genesis of this cancer, and also in cancer of the mouth which is suspected of being related to such chewing habits as betel quid, or nass, or tobacco. There is believed to be an urgent need for methods of assaying the carcinogenic effects of environmental factors.

Proposed Course: Groups of hamsters are currently fed powdered chow containing Annona muricata plant, or snuff, or arecoline, with added calcium hydroxide, to investigate their effects on the gastrointestinal tract. In another experiment, hamster cheek pouches are being treated with a known carcinogen with and without added calcium hydroxide, to ascertain any effect there may be of the added alkali. Attempts to develop a reliable method of testing the effects of suspected weak carcinogens are being continued.

Serial No. NCI 501a
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Geographic Pathology Field Study on Uterine Cancer in Negro Women in New York City and the Washington, D.C. Metropolitan Area

Previous Serial Number: Same

Principal Investigators: Lucia J. Dunham, M.D., Louis B. Thomas, M.D., and Harold L. Stewart, M.D.

Other Investigators: None

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/2
Professional: 1/2
Other: 0

The article was submitted to the Journal of the National Cancer Institute on March 8, 1972.

Serial No. NCI 501b
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: A Review of the Scientific Literature on the Subject of "Cancer in Man at the Site of Prior Benign Lesion of Skin or Mucous Membrane"

Previous Serial Number: Same

Principal Investigator: Lucia J. Dunham, M.D.

Other Investigators: None

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional:
Other:

Project Description:

Objectives and Methods Employed: To categorize the prior benign lesions: (1) Induced by known carcinogens (2) Benign lesions of a familial disease (3) Congenital lesions (4) Benign tumors (5) Lesions based on trauma or inflammation (6) Idiopathic lesions found clinically associated with cancer (7) Lesions that are manifestation of a focal neuropathy or constitutional disorder.

Major Findings: The types of injury and the pathology of the lesions are noted and discussed. Risks of cancer are described by the type and site of lesion.

Significance: To improve understanding of the natural history of some human cancers and to provide suggestions for research studies from such data.

Proposed Course: The article has been accepted for publication by Cancer Research. The project is completed.

Serial No. NCI 501c
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Effects of Gambier, an Ingredient of the Betel Quid, Administered to Mice by Various Routes

Previous Serial Number: Same

Principal Investigators: Calum S. Muir, M.D., and Lucia J. Dunham, M.D.

Other Investigators: None

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional:
Other:

Project Description:

Gambier was administered to about 500 C_3H/HeN and hairless mice by subcutaneous route, by painting and inunction, and in food and drinking water.

Tumors were not produced during the course of experiments carried out at the National Cancer Institute. Muir and Hwang, working in Singapore, have recently produced sarcomas at subcutaneous sites in mice by repeated injections of an alkaline alcoholic extract of Gambier. Dunham has tried to produce sarcomas in the loose areolar tissue beneath the pouch epithelium and also in the tissues beneath the skin of the dorsum of the hamster foot by repeated injections of an alkaline alcoholic extract of Gambier. The experiment is completed and tumors were not produced.

Work will not be continued on this project.

Serial No. NCI 501g
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Report on Histologically Diagnosed Cancers in Indians of the United States, 1950 to 1966

Previous Serial Number: Same

Principal Investigators: Gert L. Laqueur, M.D., John C. Bailar, M.D., and Lucia J. Dunham, M.D.

Other Investigators: None

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional:
Other:

Project Description:

The histologic and site diagnoses of 694 cancers in USA Indians are reported and analyzed. An article has been prepared for publication.

Serial No. NCI 504
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: An Electron Optical Investigation of Normal and Pathologic Tissue

Previous Serial Number: Same

Principal Investigator: William G. Banfield, M.D.

Other Investigators: George Kasnic, Clyde Dawe, M.D., Willie Morgan, Austin Farley, and Alan Rabson, M.D.

Cooperating Units: Biological Laboratory, National Marine Fisheries Service (NOAA), Oxford, Maryland

Man Years (July 1, 1971 - June 30, 1972)

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

Objectives: 1. To describe and interpret the ultrastructure of selected pathologic states and to compare the results with suitable controls.

2. To look for and study the inciting agent or accompanying agent in neoplastic and other disease states using human, animal and tissue culture material.

Major Findings: 1. A study of the dense granule occurring in the elementary body of the pox viruses remains to be written up.

2. Shope fibroma: Electron microscopic structure of associated lamellae was completed and published.

3. The electron microscopy of an oyster herpesvirus was completed and the manuscript submitted to Science.

4. Two lymphomatous lesions in bivalves were examined but no structures definitive of virus were found.

Significance: 1. The dense granule may be of fundamental functional importance in the development of the virus and in its effects on the host. There are indications that it may exist in other viruses outside of the pox group.

2. The unit structure we have described for the Shope fibroma lamellae may be a type of basic unit found in other membranous structures such as desmosomes.

3. Herpesvirus has been linked with human lymphomas. Whether the oyster virus affects man must be determined.

Proposed Course: 1. Write up the dense granule work. Attempt to isolate the granule for further study when time permits.

2. No further work is contemplated on the Shope lamellae or the oyster.

Publications:

1. Banfield, W.G., and Kasnic, G.: Shope fibroma: Electron microscopic structure of associated lamellae. J. Ultrastruct. Res. 37: 37-40, 1971.

Serial No. NCI 504b
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: An Investigation of the Collagenous Connective Tissues and Elastin

Previous Serial Number: Same

Principal Investigator: William G. Banfield, M.D.

Other Investigators: C.K. Lee, L. Sokoloff, M.D., W.C. Roberts, M.D., L.M. Buja, M.D., and C. Lee

Cooperating Units: NIAMD, Lab. Exp. Path., Section on Rheumatic Diseases; NHI, Cardiology Branch, Section on Pathology

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Objectives: To elucidate the normal and abnormal structure of elastin and collagen and their associated cellular and intercellular components.

Major Findings: 1. The technique for preparing ultrathin sections from coverslip cultures was worked out. Comparisons were made between chondrocytes and fibroblasts and chondrocytes and fibroblasts grown under different conditions. Technical help was lost and the project was stopped indefinitely.

2. The results of the study of long spacing collagen type fibrils with especial reference to their occurrence in normal and diseased hearts are in manuscript form.

3. The connective tissues, elastin and collagen, are continuing to be studied with the electron probe and scanning electron microscope as indicated under those headings.

Significance: The mucoprotein collagen complex as expressed in the LS type collagen fibrils may be an integral part of the connective tissue system. These fibrils are present, often in large numbers, in thyroid medullary tumors, Hodgkin's disease lymph nodes and other tumors. Their significance with respect to the metabolic state of the tissue in which they are found should be explored.

Proposed Course: 1. Publish the LS fibril manuscript.

2. Collaborate with Dr. F.C. Barter of the Heart Institute in looking at the bone changes of fibrogenesis imperfecta osseae. Specifically, to find whether the mass of coated vesicles found previously in bone sections is characteristic of the disease.

Serial No. NCI 505
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Evaluation of Potential Pulmonary Carcinogens, Asbestos, Tobacco, Silicates and Heavy Metals and their Relation to the Physiological Status of the Lung

Previous Serial Number: Same

Principal Investigator: Mearl F. Stanton, M.D.

Other Investigators: Miss E. Miller, Mrs. C. Wrench, and Miss Betty Sanders and staff

Cooperating Units: Johns-Manville Research and Engineering Center, Manville, New Jersey; Owens-Corning Fiberglass Corp., Toledo, Ohio; Dr. Fred Bock, Roswell Park Memorial Center for Cancer Research; Mel-Labs Inc., Contract NIH 70-2295

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/2
Professional: 1/6
Other: 1/3

Project Description:

Objectives: (1) Investigations into pulmonary carcinogenesis with tobacco products and other potential pulmonary carcinogens for man. (2) Investigations into the factors affecting development of mesotheliomas experimentally induced with asbestos, fibrous glass and related fibrous minerals.

Major Findings: (1) Investigations into pulmonary carcinogenesis with tobacco products and other potential pulmonary carcinogens for man: A recent and perhaps the most significant accomplishment of this unit has been the successful induction of pulmonary epidermoid carcinomas in rodents with condensates of cigarette smoke utilizing a model which permits the quantitative assessment of carcinogenic potency. The project began with an analysis of response of the rat lung to directly implanted pellets of an inert beeswax-tricaprylin mixture to which could be added potential carcinogens. Based on examination of the morphological response of the lung to conventional carcinogens such as benzo(a)pyrene and 3-methylcholanthrene in the pellets the following conclusions were ascertained. Respiratory epithelium adjacent to carcinogen containing pellets was converted promptly and consistently to a proliferating keratinized squamous epithelium in contrast to the inert granulomatous response to pellets devoid of carcinogens. This early response, which

might well serve as a rapid indicator of the potential carcinogenicity of suspected substances, progressed from simple metaplastic squamous epithelium through dysplastic epithelium with microscopic evidence of invasion to overt metastasizing epidermoid carcinomas. The incidence and time of development of the overt carcinomas and preneoplastic lesions was directly dependent on both time and dose with metaplastic conversion occurring within 4 weeks but with overt carcinomas requiring a minimum of 55 weeks of development. Experiments are being done with a variety of substances such as air pollutants, chromium, beryllium and arsenic suspected of being pulmonary carcinogens for man but of direct relevance to this report are the preliminary results with cigarette smoke condensates provided to us by Dr. Fred Bock. Within 68 weeks both crude condensate and heptane extracted condensate of cigarette smoke have yielded from 8 to 18% epidermoid carcinomas with doses of 12 to 69 mgms incorporated in the pellets and virtually all of the pellets show the initial metaplastic response. In contrast non-burnt tobacco has induced a 2% incidence of metaplastic change in the same time period and controls such as the non-carcinogen anthracene and tobacco ash show no metaplastic alteration. A few experiments which have continued for longer periods show a much higher incidence of carcinomas with tobacco smoke condensate. Thus we believe that this method may prove to be a reliable means of quantitating the response of the lung to those fractions of tobacco smoke condensate that are critical to pulmonary carcinogenesis, and may serve as the best means of detecting and eliminating by substitution of these fractions in cigarettes.

(2) Investigations into the factors affecting development of mesotheliomas experimentally induced with asbestos, fibrous glass and related fibrous minerals: A recent paper (JNCI 48: 797-821, 1972) summarizes past investigations into mechanisms of fiber carcinogenesis. Seventeen potential carcinogens (3 types of asbestos in 7 forms, 6 types of fibrous glass, 2 types of silica, and 2 types of metal particles) were applied on a fibrous glass vehicle directly to the pleura of rats. In 2 years amosite, chrysotile, and 3 different specimens of crocidolite yielded equally high incidences of pleural mesotheliomas. Hand-milled crocidolite ore not exposed to extraneous oils or metallic milling yielded dose-related tumor responses comparable to those of conventionally milled asbestoses. Neither pulverized fragments of the steel mill nor nickel metal at doses exceeding potential contaminating levels yielded tumors. The intact fibrous glass vehicle did not induce mesotheliomas nor did its absence alter the incidence of crocidolite-induced tumors. However, when the fibrous glass vehicle and two other types of fibrous glass of relatively large diameter were reduced to smaller fibrous fragments and applied to the pleura a few mesotheliomas were observed. Similarly, 1 of 2 samples of non-fibrous silica yielded 1 mesothelioma. Two forms of an especially fine fibrous glass, further milled to approach the size of asbestos particles, induced moderately high incidences of mesotheliomas. Notably, the crocidolite inducing a high incidence of mesotheliomas, induced fewer mesotheliomas when reduced to a much finer fiber by excessive milling. These observations suggest that the carcinogenicity of asbestos and glass is related to structural features rather than acquired or intrinsic physiochemical properties. Of importance is the evidence that fibrous glass of particularly fine diameter is carcinogenic in the rat and may be so in man. In fact current experiments strongly suggest

that any durable fiber in the size range of asbestos fibers may be carcinogenic. Whether they can be altered by affecting durability or surface structure is being studied. To our knowledge, only limited work is being done outside this laboratory on the mechanisms of asbestos carcinogenesis. These experiments like those with tobacco condensate require a minimum of 2 years observation but currently seem to be the only means to a definitive answer.

Proposed Course: Attachment 1 is a list of ongoing long-term animal experiments covering essentially the experiments with pulmonary response to tobacco smoke condensate pellets and pleural response to fibrous minerals. Future plans are entirely dependent on available space for animal housing. Since all of the experiments require 2 years or more observation, a relatively high ratio of animal facilities to investigator time is essential. This in part is managed through current contract arrangements with Meloy Laboratories (contract NIH 71-2284). The recent successful results particularly the induction of epidermoid carcinomas of the lung with cigarette smoke condensate, point to an urgent need to expand these facilities so that this valuable lead can be exploited. At the same time experiments to investigate the carcinogenicity of mineral fibers and to gain further insight into their mechanism of action have been planned to occupy most of the present space in the next 3 years and should not be neglected because of the direct implications to man.

Briefly our plans call for testing various fractions of cigarette smoke condensate and the condensate of cigarette smoke from cigarettes with altered or reduced tar and nicotine content as well as extracts from specific air pollutants and other potential pulmonary carcinogens that may be lipid soluble. Work with fibers involves the tedious assessment of fiber sizes and separation of fibers into specific size groups for testing utilizing asbestoses, glasses, aluminum oxide, silica, iron and carborundum fibers. The effects of reduction in durability as a result of pyrolysis and the effect on surface properties by fiber coating will be investigated as time and space permit. Electron microscopy of candidate specimens of fibers and fine structure analysis of the early metaplastic alterations in pulmonary epithelium are in progress with the aim of understanding the preneoplastic changes that may be related to the induction of cancer.

Publications:

1. Stanton, M.F., and Wrench, C.: Mechanisms of mesothelioma induction with asbestos and fibrous glass. J. Nat. Cancer Inst. 48: 797-821, 1972.

2. Stanton, M.F., Miller, E., and Wrench, C.: Experimental induction of epidermoid carcinomas of the lung with cigarette smoke condensate. J. Nat. Cancer Inst. (in press).

ATTACHMENT I (1)

Current Ongoing Experiments January 1972	DOSE	DATE OF TREATMENT	NO. SURVIVING TREATMENT	DEAD OR KILLED	NO. SURVIVORS
PLEURAL RESPONSE TO:					
CROCIDOLITE ASBESTOS					
Unopened fibers, Cape Blue cobbed ore	40 mg	5-27-70-6-01-70	30	6	24
Fully pulverized UICC Crocidolite	40 mg	3-17-71	30	0	30
UICC Crocidolite - 2nd Series	40 mg	4-08-71	30	2	28
UICC Crocidolite - 2nd Series	20 mg	4-09-71	30	0	30
UICC Partially pulverized Crocidolite 2nd Series	40 mg	4-16-71	30	0	30
Microfibrillar fraction - UICC Crocidolite	40 mg	5-04-71	30	1	29
Macrofibrillar fraction - UICC Crocidolite	40 mg	1-06-72	29	0	29
J-M finely ground Crocidolite #4106-23-13	40 mg	5-05-71	30	0	30
J-M long, fine Crocidolite $>10\mu$ x $.5\mu$ #4106-22-4	40 mg	5-07-71	30	3	27
J-M short, fine Crocidolite $<10\mu$ x $.5\mu$ #4106-25-2	40 mg	5-06-71	30	2	28
CHRYSOTILE ASBESTOS					
Air-Cleaned Jeffrey Chrysotile J-M 6D20	40 mg	7-22-70	30	5	25
Air-Cleaned Jeffrey Chrysotile J-M 6D20	20 mg	7-31-70	30	16	14

ATTACHMENT I (Cont'd) (2)

Current Ongoing Experiments January 1972	DOSE	DATE OF TREATMENT	NO. SURVIVING TREATMENT	DEAD OR KILLED	NO. SURVIVORS
Air-Cleaned Jeffrey Chrysotile J-M 6D20	10 mg	8-02-70	30	8	22
Air-Cleaned Jeffrey Chrysotile J-M 6D20	5 mg	8-04-70	30	5	25
Air-Cleaned Jeffrey Chrysotile J-M 6D20 Fully pulverized	40 mg	11-23-70	30	9	21
Air-Cleaned Jeffrey Chrysotile J-M 6D20 Partially pulverized	40 mg	7-09-71	30	0	30
Cobbed Crude Chrysotile ore from Jeffrey mines	40 mg	4-13-71	30	0	30
FIBROUS GLASS					
Whole fiber uncoated fine fiberglass, Owens-Corning	40 mg	4-14-70	30	8	22
Glass fibers $<5\mu$ x 1μ, J-M	40 mg	8-31-70	30	3	27
Glass fibers $<5\mu$ x 3μ, J-M	40 mg	9-06-70	30	3	27
Glass fibers $>10\mu$ x 1μ, J-M	40 mg	9-13-70	31	3	27
Glass fibers $>10\mu$ x 3μ, J-M	40 mg	9-19-70	30	4	26
J-M Code 106 Microfiber glass	40 mg	12-01-70	30	10	20
Sheared uncoated 0-C fine fiberglass	40 mg	12-30-70	30	2	28
Sheared uncoated 0-C fine fiberglass	80 mg	12-23-70	30	3	27

ATTACHMENT I (Cont'd) (3)

Current Ongoing Experiments January 1972	DOSE	DATE OF TREATMENT	NO. SURVIVING TREATMENT	DEAD OR KILLED	NO. SURVIVORS
Sheared uncoated 0-C fine fiberglass	160 mg	1-15-71	30	4	26
Glass fibers, long, fine J-M #4106-27-5, Code 100	40 mg	5-13-71	30	9	21
0-C fine uncoated fiberglass fully pulverized	40 mg	8-24-71	30	0	30
0-C fine uncoated fiberglass fully pulverized	40 mg	8-02-71	30	2	28
J-M microfiber glass ⌀.05μ x .2μ (Short) #4106-23-7	40 mg	8-26-71	30	0	30
Yellow fiberglass, pad controls	80 mg	10-08-71-10-14-71	64	1	63
RELATED FIBROUS AND NON-FIBROUS MATERIALS					
Antigorite	40 mg	7-13-70	30	13	17
Aluminum Oxide Whiskers	40 mg	9-30-70	30	8	22
Aluminum Oxide Whiskers	20 mg	10-12-70	30	3	27
Aluminum Oxide Whiskers	10 mg	10-19-70	30	8	22
Aluminum Oxide Whiskers	5 mg	10-20-70	30	5	25
Linde Corp. Alumina 0.3μ	40 mg	12-18-70	30	2	28
Attapulgite #4418-100-1	40 mg	5-12-71	30	1	29
Tungsten Carbide Whiskers	40 mg	5-24-71	30	7	23

ATTACHMENT I (Cont'd)

Current Ongoing Experiments January 1972	DOSE	DATE OF TREATMENT	NO. SURVIVING TREATMENT	DEAD OR KILLED	NO. SURVIVORS
PULMONARY RESPONSE TO PELLETS:					
TOBACCO AND TOBACCO CONTROLS					
Beeswax-tricap controls 1:1	.05 cc	1-23-70-2-06-70	44	20	24
Beeswax-tricap controls 1:1	.05 cc	3-17-70	10	1	9
Beeswax-tricap controls 1:1	0.1 cc	5-12-70-6-05-70	50	10	40
Crude tobacco and beeswax 1:1	.05 cc	2-17-70-3-11-70	110	51	59
Heptane soluble tobacco fraction & beeswax 1:1	.05 cc	3-08-70-4-14-70	100	34	66
Heptane soluble tobacco fraction & beeswax 1:3	.05 cc	4-24-70-5-07-70	50	24	26
Heptane soluble tobacco fraction & beeswax 1:1	0.1 cc	5-14-70-5-28-70	51	21	30
Heptane soluble tobacco fraction & beeswax 3:1	0.1 cc	6-11-70-6-17-70	50	21	29
Heptane soluble tobacco without beeswax	.05-.08 cc	12-21-70 1-07-71	32	1	31
Anthracene & beeswax-tricap 1:1 40 mg/cc	0.1 cc	3-12-71	28	0	28
TOBACCO AND FIBROUS MATERIALS					
Asbestos & tricap 200 mg/3cc	.08 cc	12-17-70	30	4	26

ATTACHMENT I (Cont'd)

Current Ongoing Experiments January 1972	DOSE	DATE OF TREATMENT	NO. SURVIVING TREATMENT	DEAD OR KILLED	NO. SURVIVORS
Asbestos & heptane soluble tobacco 200 mg/3cc	.08 cc	12-08-70-12-14-70	30	5	25
Fiberglass & tricap 100 mg/cc	0.1 cc	12-03-70	26	4	22
Fiberglass & heptane soluble tobacco 100 mg/cc	0.1 cc	11-24-70	30	10	20
Crude tobacco & asbestos with tricap 240 mg/cc 2:3	0.1 cc	2-12-71-2-22-71	30	4	26
Crude tobacco & fiberglass with tricap 100 mg/cc 2:3	0.1 cc	2-25-71-3-05-71	30	3	27

Serial No. NCI 514
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Induced and Spontaneous Neoplasms in Laboratory Animals. Factors that Influence their Incidence and the Chemical Changes Induced by the Neoplasms

Previous Serial Number: Same

Principal Investigator: Ambadas S. Mulay, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

A. Objectives: Induction of glandular stomach lesions in guinea pigs.

Methods Employed: A group of male guinea pigs of Hartley strain is given by stomach tube, suspension of nitrosomethyl urethane 3 times a week and another group receives the suspension by intraperitoneal route once a week. Animals are examined every day for their general health and possible initiation of tumors.

Major Findings: Experiments are still in progress. No findings to report at this time.

Significance: A reliable method of producing high incidence of gastric carcinoma will give us a tool to study the chemistry of the lesion and may be useful in determining relative effectiveness of various chemotherapeutic agents.

Proposed Course: The work will continue in the next fiscal year.

B. Objectives: Induction of glandular stomach lesions in rats.

Methods Employed: Male rats of Osborne-Mendel strain and male and female rats of Wistar strain have been started on N-methyl-N-nitroso-N-nitroguanidine in drinking water.

Major Findings: Tumors have been observed in the glandular stomach of three Osborne-Mendel rats and one Wistar rat. Their histology is under study.

Significance: Induction of glandular stomach tumors could lead to development of a transplantable stomach tumor. This could prove to be an interesting experimental tool.

Proposed Course: The results on this group of animals were not satisfactory, as the location of the laboratory was moved and many animals suffered from exposure and colds. The experiments are being repeated and will continue into the next year.

C. Objectives: Study of alkaline phosphatase isozymes of liver and hepatoma tissue.

Methods Employed: Osborne-Mendel rats are fed powdered Purina chow diet containing N,N-dimethyl-p-(m-tolylazo)aniline. When hepatomas are induced, tumor tissue, adjacent liver tissue, and liver tissue in control rats are examined for alkaline phosphatase isozyme which is inhibited by L-phenylalanine.

Major Findings: Experiments are just starting. No findings.

Significance: It was reported from this laboratory in 1952 that alkaline phosphatase was elevated in serum and tumor tissues of hepatoma-bearing rats. As alkaline phosphatase is elevated in various pathological conditions, no significance was attached to the finding. Now, W. Fishman of Tuft University Pathology Department, intimates that alkaline phosphatase isozyme, inhibited by L-phenylalanine, is increased in hepatoma-bearing animals and it may be the same as α-fetal globulin. It would be interesting to verify the observations and check the relationships.

Proposed Course: Work will continue in the next fiscal year.

D. Objectives: Induction of lung tumors in rats.

Methods Employed: Osborne-Mendel rats are tube-fed twice a week with freshly prepared solution of 4-nitroquinoline-1-oxide in 10% dimethylsulfoxide. Rats are examined at regular intervals for signs of tumors.

Major Findings: Few forestomach tumors were observed in animals that came to autopsy. No lung tumors have been observed thus far.

Significance: Transplantable lung tumors could be used as an interesting experimental tool.

Proposed Course: Work will be discontinued when these experiments are finished.

Publications:

1. Mulay, A.S., and O'Gara, R.W.: Effect of diet on N,N-dimethyl-p-(m-tolylazo)aniline carcinogenesis in rats. Proc. Soc. Exp. Biol. Med. 137: 907-910, 1971.

Serial No. NCI 516c
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Viral Carcinogenesis. The Murine Sarcomagenic Viruses: Problems of Immunity, Resistance, and Pathogenesis and their Relation to Cell Structure

Previous Serial Number: Same

Principal Investigator: Mearl F. Stanton, M.D.

Other Investigators: Miss E. Miller, Mrs. C. Wrench, and Miss Betty Sanders and staff

Cooperating Units: Lloyd Law, M.D. and staff (NCI 3200), Richard Adamson, M.D. and staff (LCP)

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/2
Professional: 1/6
Other: 1/3

Project Description:

Objectives: (1) Collection and development of competence in special areas of morphology. (2) Studies of histopathological and fine structure alterations related to the pathogenesis of disease induced by a murine sarcoma virus.

Major Findings: An ongoing program has been the development of special competence in the histopathological and fine structural aspects of tumors of the lung, bone and mammary glands of rodents. Collection and examination of induced and naturally occurring bone tumors has been in progress for more than 5 years and will this year lead to the publication of a chapter on bone tumors in mice in the current series of WHO monographs on the "Pathology of Neoplasms in Experimental Animals". Our interest in bone tumors stems from the induction of bone tumors in rodents with chemical carcinogens several years ago. A series of these tumors ranging from Ewing's type to osteogenic sarcomas are available as transplantable tumors and are frequently supplied to other investigators. Currently, Dr. Richard Adamson, Laboratory of Chemical Pharmacology is studying the effects of bone seeking heavy metal chemotherapeutic agents on the osteogenic varieties.

The collection and development of competence in lung tumors is essential for the work previously described and additionally serves as an aid to other

investigators in the area and to the NCI Registry of Experimental Cancers. A current project on which Mrs. Wrench serves as the primary investigator is the study of the fine structure of the early squamous metaplasia preceding carcinoma at the site of implanted carcinogen pellets and the comparison of this change with the metaplasia associated with non-specific pulmonary inflammation which does not progress to cancer.

Interest in mammary neoplasia in rats is an offshoot of current long-term pulmonary experiments. Since over 50% of female strain OM rats develop mammary tumors in the second year of life it is necessary to surgically remove these in our experimental groups. As a result we have correlated data on incidence, growth rate and morphology in several hundred excised mammary neoplasms.

Our interest in the morphology and fine structure of tumors induced by murine sarcoma viruses continues along lines of previous publications. The natural history and origin of these tumors particularly those of bone in the rat have received exhaustive study. Recently interest has centered on the morphology of cultured mouse embryo cells of line 3T3 which following infection with MSV (Moloney) show modulations in structure to an epithelioid type with fine structural characteristics previously identified as unique hallmarks of infection with this virus.

Experience gained from examining neoplasms induced by murine sarcomagenic viruses strongly suggests that all of the neoplasms may have a common cell of origin. If this is the case, the most likely candidate would be a primitive cell of the reticuloendothelial-histiocytic series. Derivation of all of the neoplasms from cells closely related to lymphoreticular neoplasms would of course be most significant to the phenomenon of C-type virus oncogenesis.

As previously reported, the disease induced by this virus in both rats and mice is initially an inflammatory granuloma in which atypical cells arise. This response occurs in many tissues although definite tissue-selectivity occurs. Ultimately a small percentage of the lesions evolve into simple neoplasms. The unique reactive response to either the atypical cells or the murine sarcoma virus and its relation to leukemogenesis and such neoplasms in man (for example, Kaposi's disease, Hodgkin's disease and the reticuloendotheliosis) that show similar reactive components make this disease a particularly critical one in studies of viral carcinogenesis. Further studies in viral oncogenesis have centered on characterization of cell structure and cell alterations induced by infection with so-called "murine sarcoma virus" by electron microscopy.

Proposed Course: The current problem is one of seeking ways of clearly and unequivocally establishing the progenitor cell type of the neoplasms. No simple means is at hand and investigations may be deferred for some time unless a reasonable approach to this problem can be devised. Nevertheless, the electron microscopy, histology and distributional analysis of lesions from several hundred rats are being analyzed. Of particular interest is our effort to compare changes induced in the tumors of osseous and neurogenic tissues

with those in the transplantable MSB-1. Analysis of tissue morphology by a variety of means will probably occupy the better part of the year.

Serial No. NCI 519
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Induction of Neoplasms, Selection of Transplantable Strains of Tumors and Study of Tumor and Host Chemistry

Previous Serial Number: Same

Principal Investigator: Ambadas S. Mulay, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

A. Objectives: To induce glandular stomach tumors in rats and to establish a transplantable strain of these tumors.

Methods Employed: Male rats of Osborne-Mendel strain and male and female rats of Buffalo strain are fed a semisynthetic diet deficient in vitamin A. To this diet is added a carcinogen, 2,7-diacetamidofluorene, suspended in corn oil. Other groups of rats are fed the same diet except for the addition of an irritant - celite. There are control groups without carcinogen and without irritant. Diets are thoroughly mixed with mechanical mixer and stored at $0^{o}C$. Fresh diet is prepared every other week. Animals are regularly examined for their general health and possible tumors.

Major Findings: The experiments are still in progress. Animals that came to autopsy thus far did not show any striking changes in glandular stomach. Few liver lesions and few subcutaneous tumors were observed.

Significance: This experimental study, when successful, may throw some light on the mechanism of induction of glandular stomach tumors. Successful development of transplantable tumor would give us a tool for further study of this neoplasm.

Proposed Course: The study will continue in the next year until the experiments are terminated.

B. Objectives: To induce glandular stomach tumors in rats and to establish a transplantable strain of these tumors.

Methods Employed: Synthesize the chemical, 4-hydroxyaminoquinoline-1-oxide. Feed the compound in food, in water, or by stomach tube to Osborne-Mendel, Fischer, Marshall, and Wistar rats. Examine the animals for tumors at regular intervals. Transplant the primary tumors as they become available and try to establish a transplantable tumor line.

Major Findings: The first series of experiments did not yield any satisfactory results - most of the animals died within 6 months and only one forestomach tumor was observed. The experiments are being repeated again with certain modifications.

Significance: Transplantable glandular stomach tumor could be an interesting experimental tool for studying the characteristics of this tumor.

Proposed Course: The study will continue through the next year.

C. Objectives: To induce stomach tumors in rats and to establish a transplantable strain of these tumors.

Methods Employed: A suspension of nitrosomethyl urethane in 20% ethanol is fed by a tube to Osborne-Mendel rats once every week. Animals are examined every day for their general health and periodically checked for signs of tumor induction on gross examination.

Major Findings: Forestomach polyps were found in some of the rats that came to autopsy and glandular stomach tumors suspected in others. Experiments are still in progress and microscopic examination of the lesions has not yet been made.

Significance: Transplantable glandular stomach tumor could be an interesting experimental tool for the study of these tumors.

Proposed Course: The study will continue through the next year.

Serial No. NCI 521a
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Investigations of Mechanisms of Tumor Induction by Polyoma Virus in Mice

Previous Serial Number: Same

Principal Investigator: Clyde J. Dawe, M.D.

Other Investigators: W.D. Morgan

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional:
Other:

Project Description:

Objectives: This continuing project is designed to clarify the conditions necessary for and the cellular activities leading to tumor development in mouse salivary glands infected with polyoma virus. Previously published works from this project have shown the following:

1. In PV-infected organ cultures of salivary glands, morphologic transformation occurs, closely resembling tumor development in vivo. When such transformed cultures are transplanted to mice, typical salivary gland tumors appear at the site of transplant.

2. In the organ culture system, the visible sequence of events is: (a) Lytic infection of mesenchyme; (b) Lytic infection of epithelium; (c) Emergence of transformed cells, some of which undergo viral lysis while others proliferate.

3. At very early stages of embryonic development, salivary gland or pre-salivary tissue does not undergo neoplastic transformation subsequent to PV infection.

4. When the epithelial and mesenchymal components of embryonic salivary glands are isolated from each other by trypsin dissection neither component is able to give rise to a neoplasm after infection with PV.

5. Recombination of salivary epithelium with salivary mesenchyme restores the capacity for tumor development to occur. Recombination of salivary epithelium with tooth bud mesenchyme, or of tooth bud epithelium with salivary gland mesenchyme, does not restore the capacity for tumor development.

6. PV-induced tumor tissue can substitute for normal salivary gland mesenchyme in supporting morphogenesis in vivo. In vitro, the tumor tissue has so far been observed to support growth, but not morphogenesis of salivary epithelium.

7. Epithelial origin of the PV-induced salivary gland tumors has been demonstrated. This was done by reassembling glands from $T6T6$ chromosome-marked epithelium and unmarked mesenchyme, then determining the presence or absence of the marker chromosomes in tumors induced by PV in the reassemblies.

Specific objectives are as follows:

1. During the past year, efforts were directed mainly toward using the trans-filter system to determine whether neoplastic transformation could be induced in rudimentary salivary epithelium in the absence of interaction with salivary mesenchyme, but in the presence of polyoma-induced salivary tumor cells across the membrane. The controls for this experiment are also of special interest, as it is desired to learn whether normal salivary epithelium can be induced to become neoplastic through trans-filter interaction with polyoma tumor cells in the absence, as well as in the presence of infectious virus.

2. Experiments were continued with combinations of polyoma salivary tumors (lacking infectious virus) and normal salivary epithelium, and of the same tumors combined with normal mammary rudiment epithelium, in vivo. This work is being done to determine the specificity of the ability of polyoma salivary tumors to support growth and morphogenesis of normal epithelium from different organs.

Methods Employed: In (1) above, isolated salivary epithelium from normal $C3H/Bi$ mouse embryos of 13 to 14-days' gestation is placed on one side of a millipore filter, while polyoma salivary tumor cells of line 9391 are grown on the opposite side. After 2 to 3 days, when the salivary epithelium has become attached and spread out to form a monolayer, polyoma virus is added to the culture. Cultures of this type are then carried for periods up to 2 months to follow the growth and morphological characteristics of the epithelium. Control cultures consist of similar configurations using normal salivary mesenchyme opposite normal salivary epithelium, and mammary epithelium opposite salivary mesenchyme. Salivary epithelium alone is also used as a control, and all four of these types of culture will eventually be studied both with and without polyoma virus infection.

Evaluation of results is based on (1) microscopic examination of fixed-stained preparations of the trans-filter cultures and (2) transplantation of the salivary epithelial cells to syngeneic newborn mice to determine

tumorigenicity and histologic characteristics of the tumors induced, if any. To be able to determine whether contamination by 9391 cells of the epithelium under test has occurred, we will use $T6T6$ chromosome-marked salivary epithelium. Tumors arising from this epithelium should carry the $T6T6$ markers, while those originating from line 9391 would not.

In (2) above, normal rudimentary epithelium from 16-day mammary rudiments or from 13-14 day salivary rudiments are placed within a small piece of transplantable salivary tumor and transplanted subcutaneously to syngeneic newborn mice. After 12-14 days, the transplants are removed and serial-sectioned to determine whether morphogenesis has occurred in the epithelium within the tumor.

Major Findings: These experiments have progressed slowly because of the high frequency of technical mishaps that occur at various points in the tedious procedure. At this time, all our information is on the controls, as no cultures of salivary epithelium trans-membrane to 9391 tumor cells in the presence of virus have been carried to completion.

We have thus far observed:

1. In absence of virus, normal salivary epithelium trans-filter to normal salivary mesenchyme grew well for periods longer than 1 month, but did not undergo morphological transformation. In the absence of trans-filter salivary mesenchyme, salivary epithelium failed to sustain itself beyond 2-3 weeks.

2. In the absence of virus, normal salivary epithelium trans-filter to 9391 tumor cells grew well for periods longer than 1 month, but did not undergo morphological transformation.

3. In presence of virus, salivary epithelium trans-filter to normal salivary mesenchyme grew for a week to 2 weeks, then became static, after which it resumed growth. In this second phase, little mesenchyme persisted on the opposite side of the membrane, as it had undergone viral lysis. Morphologically, the epithelium contained many cells with enlarged, abnormal appearing nuclei, but the entire population did not appear to be transformed. Since we have had only 1 culture reach the 30-60 day period in this category, more cultures of the same type will have to be observed before general conclusions can be reached.

4. An interesting by-product of the experiment was noted. Line 9391 tumor cells when infected with polyoma virus showed no sign of viral lytic effect or impairment of growth. However, viral infection was established, since 88 days after infection the culture fluids were found to have a high polyoma tumor-inducing capability in newborn mice. Of particular interest in the small number of test mice used was the occurrence of multiple mammary tumors and the absence of any gross salivary gland tumors. Thus, passage of our strain of virus through 9391 salivary tumor cells seemed to increase its oncogenicity for mammary gland and decrease its oncogenicity for salivary gland.

5. In the second type of experiment designed to observe the presence or absence of morphogenetic response of mammary rudimentary epithelium within salivary tumors, no progress has been made. During serial passage of the two tumor lines previously used in this work, both lines progressed into distinctly epithelial types from their previously mesenchymoid histologic character. This made them unusable for this experiment, and we are now trying to find suitable new tumors of PV-induced salivary gland origin to continue this work.

Significance: On the basis of the previous work summarized under Objectives, we have postulated (publications cited below) that the genesis of PV-induced salivary tumors depends, like normal salivary organogenesis, on interaction between salivary epithelium and its mesenchymal stroma, at least during a critical initial period. Following this critical period, virus-transformed epithelium becomes independent of salivary mesenchyme for continued growth, and in fact behaves as if it produced its own growth-sustaining materials, previously supplied by mesenchyme. The experiments outlined above will further test this concept. If the concept can be confirmed biologically, biochemical investigations to determine the nature of the growth-sustaining factor(s) will be indicated. The ability of epithelial cells, under the influence of oncogens, to "turn on" synthesis of growth-sustaining factor(s) normally controlled by stroma may be a fundamental principle of carcinogenesis in general.

Proposed Course: It is planned to complete the experiments as outlined. However, if the technical problems currently impeding progress are not resolved in the next few months, consideration will be given to devising new techniques to answer the same questions by other approaches.

We have designed and are carrying out a small experiment to check the repeatability of the observation that polyoma virus propagated on 9391 tumor cells induces mammary tumors at an increased frequency and salivary tumors at a decreased frequency, as compared with virus grown on P388D1 mouse lymphoma cells.

Publications:

1. Dawe, C.J.: Changes in cell interrelationships during epithelial carcinogenesis. In Takayama, S. and Odashima, S. (Eds.): Topics in Chemical Carcinogenesis. Tokyo, University of Tokyo Press (in press).

2. Dawe, C.J.: Epithelial-mesenchymal interactions in relation to genesis of polyoma virus-induced tumours of mouse salivary gland. In Tarin, D. (Ed.): Tissue Interactions in Carcinogenesis. London, Academic Press Inc. (in press).

Serial No. NCI 538
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: A Study of Phylogenetic Aspects of Neoplasia

Previous Serial Number: Same

Principal Investigator: Clyde J. Dawe, M.D.

Other Investigators: J.C. Harshbarger, Ph.D.; W.G. Banfield, M.D.; D. Small, M.D. and others

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional:
Other:

Project Description:

Objectives: The continuing major objective of this project is to perform comparative studies of neoplasia in invertebrate and lower vertebrate animals, in order to gain a comprehensive view of the biology of neoplasia throughout the various animal phyla. This is done by collecting neoplasms and studying their morphology and natural history. Laboratory studies concerned with induction and transplantation of neoplasms in lower animals are also conducted. Phenomena such as regeneration, dedifferentiation, autotomy, molting, metamorphosis, graft rejection, and so on are studied in relation to the biology of neoplastic disease. The project also includes collection of literature concerned with neoplasms of invertebrate animals.

Major Findings: 1. The collaboration with Drs. Maire F. Mulcahy and G. Winqvist in a morphological study of the lymphoma of northern pike (Esox lucius) in Ireland was completed with the publication cited in last year's report.

During the past year the Principal Investigator has served in a consulting capacity to many outside investigators who have submitted neoplasms of fish and invertebrate animals for histological classification. Among these were plasma cell neoplasms with accompanying plasma cell leukemia in brown bullheads, experimentally induced by cell-free filtrates of the muskellunge lymphoma that is epizootic in Ontario, Canada; lymphosarcoma spontaneously occurring in a feral specimen of white catfish from southeastern Maryland; lymphoid leukemia in a cutthroat trout from Montana; oral papillomas in

cunners, a marine teleost fish, from Long Island Sound; cutaneous papilloma on the fin of a long-nosed garfish from South Carolina; a polypoid tumor of the vesicular connective tissue of the pericardium in a rock oyster from New Zealand; a disseminated sarcoma in an American oyster from the Chesapeake Bay; several neoplasms of indeterminate origin in clams (Macoma balthica) from tidal tributaries of Chesapeake Bay; bone tumors in croakers from coastal waters of Delaware and New Jersey; subcutaneous sarcomas in land-locked salmon in Japan; adenomatous polyps of the gastric mucosa in land-locked salmon in Japan; subcutaneous sarcoma in a wall-eyed pike from Deep Creek Lake, Maryland; and many others. All of these specimens have been catalogued in the Registry of Tumors in Lower Animals, for which the Principal Investigator is Project Officer.

2. Tissue culture studies of a chondrosarcoma in a corn snake continue, in collaboration with Dr. David Small of the NIDR Laboratory of Microbiology and Immunology, and Dr. William G. Banfield of the Laboratory of Pathology. The cultures have now been maintained continuously for 16 months. At last examination several months ago, cells previously observed containing electron-dense cytoplasmic particles arranged in lattices could no longer be found. Neither have virus particles been seen on repeated electron microscopic examination of these cells.

3. In search of further examples of the cholangiomas found in livers of white suckers (Catastomus commersonii) from Deep Creek Lake, Maryland, samples of this species have been collected from several other waters, in addition to another recent sample from Deep Creek Lake. No tumors have been found in any of these specimen collections.

4. The Principal Investigator continues as Chairman of the UICC Committee on Comparative Oncology. This committee is engaged in a survey of tumors in filter-feeding and bottom-feeding aquatic animals, whose objective is to determine whether these animals may be useful as indicators of environmental carcinogens. New neoplasms that have been found include oral papillomas in cunners, bone tumors in croakers, and lymphosarcoma in a white catfish. In pursuit of more information on the bone tumors in croakers, samples of these fish from different sources are being screened by skeletal X-ray examinations. This method appears promising for detection of small, presumably early tumors.

5. A Workshop on the Comparative Pathology of Hematopoietic and Lymphoreticular Neoplasms was conducted in co-chairmanship with Dr. Costan W. Berard on May 10-14, 1971. The specimen material included leukemias and lymphomas in species ranging from mollusks to man. Histopathologic study sets of the materials presented are now on loan from the Registry of Experimental Cancers of NCI. A report of the workshop and a listing of the neoplasms and related diseases in the study sets was published in Reference 1 of this report.

Significance: These investigative and service activities add to the broad base of knowledge of neoplasms in animals. They contribute to the descriptive phase of cancer research in lower animals, which has received little attention from the main body of cancer researchers. Probably the chief

value of this type of information is to enable cancer investigators to grasp a fuller understanding of cancer biology and to see the problem of cancer in its full context rather than as an isolated disorder or group of disorders of importance only to man and the higher vertebrates.

These studies are also useful in providing data and materials that unforeseeably find uses in evaluating important theories that are proposed concerning neoplasia. For example, it has been contended by R.A. Good that the apparent paucity of neoplasms in invertebrate animals supports his theory that the thymic system of cellular immunity developed evolutionarily as a mechanism to protect against malignant adaptation of cells. Another example is the oncogene theory, elaborated by R. Huebner, according to which the RNA tumor viruses evolved far back in evolution because of survival advantages afforded the host. The collection of tumors in cold-blooded vertebrate and invertebrate animals offers opportunities to obtain evidence on this concept, by looking for C-type RNA viruses and their reverse transcriptases.

A more practical potential value of these studies is that, by revealing epizootics of neoplasia in particular animals in particular locations, they may lead to discovery of environmental carcinogens. A proven past example of this application is the occurrence of hepatomas in trout fed diets contaminated with aflatoxins.

Proposed Course: The studies will be continued along the outlines given above. A report is in preparation on Neoplasms in Feral Fishes: Their Significance to Cancer Research. A chapter on Comparative Neoplasia is also in preparation for a textbook entitled Cancer Medicine, edited by E. Frei and J. Holland.

Publications:

1. Dawe, C.J., and Berard, C.W.: Workshop on comparative pathology of hematopoietic and lymphoreticular neoplasms. J. Nat. Cancer Inst. 47: 1365-1370, 1971.

2. Harshbarger, J.C., and Dawe, C.J.: Hematopoietic neoplasms in invertebrate and poikilothermic vertebrate animals. In Dutcher, R. (Ed.): Comparative Leukemia Research, 1971. Basel, S. Karger (in press).

Serial No. NCI 560
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Electron Probe and Scanning Electron Microscope

Previous Serial Number: Same

Principal Investigator: William G. Banfield, M.D.

Other Investigators: Kurt Heinrich, M.D. and staff of the National Bureau of Standards

Cooperating Units: National Bureau of Standards

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Objectives: Application of electron probe and ion probe to the analysis of biological specimens.

Major Findings: 1. We are actively engaged in trying to secure funds and personnel to start a significant collaborative program with the National Bureau of Standards in July.

2. By superimposing the strip chart record of an electron probe analysis over a photomicrograph of the tissue analyzed, we can obtain an exact intracellular localization of the points analyzed.

3. We have been able to positively identify normal elastin in 5 μ paraffin sections using the scanning electron microscope.

Significance: 1. We have taken the first positive step which should lead to applying the extensive knowledge, experience and instrumentation within the National Bureau of Standards to the electron probe and to the ion probe analysis of biological specimens.

2. The method of analysis using strip chart records combined with the actual photomicrographs magnified to exactly correlate with the strip chart analysis, opens the way to an *in situ* cell by cell analysis of differentiating tissues. Such analyses will be used to compare the normal cells with the cancerous and precancerous cells in the same section.

3. The identification of elastin in the scanning electron microscope makes possible the study of morphologic changes in this connective tissue element during aging and in pathologic conditions and makes possible the centering of the electron beam over the elastin for elemental x-ray analysis and for the study of cathode luminescence.

Proposed Course: 1. In collaboration with the National Bureau of Standards, continue to work out quantitative analytical methods for biology using the electron probe and ion probe.

2. Work out the morphology of elastin, normal and pathologic, using the scanning electron microscope.

3. Adapt the scanning electron microscope to elemental analysis with emphasis on thyroid, thyroid tumors, elastin and collagen.

4. Investigate cathode luminescence of elastin. To do this it appears essential to incorporate good transmission light microscope optics into the scanning microscope so that the cathode luminescence can be seen directly. Engineering is now working on a design for this.

Serial No. NCI 561
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Collaborative Research and Other Activities

Previous Serial Number: Same

Principal Investigator: Mearl F. Stanton, M.D.

Other Investigators: None

Cooperating Units: SVCP, SALES, JNCI

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/2
Professional: 1/2
Other: 0

Project Description: (Other Activities)

Fifty percent of the normal work week is spent as Scientific Editor of JNCI. Ten percent of the normal work week is spent in administrative activities related to contracts with the Special Virus Cancer Program, Comparative Pathology Contract with the Smithsonian Institution, and long-term animal holding facility contract with Melpar, Inc.

Proposed Course: These activities will probably continue. In addition, time will be devoted to training in electron microscopy, preliminary investigations in the study of neoplasia in lower forms of animal life, and preparation of a monograph on bone tumors in mice.

Publications:

1. Harshbarger, J.C., Cantwell, G.E., and Stanton, M.F.: Effects of N-nitrosodimethylamine on the crayfish, Procambarus clarkii. Proc. 4th International Colloquium on Insect Pathology, Soc. for Invertebrate Pathology, College Park, Maryland, 1971, pp. 425-430.

Serial No. NCI 579
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Action of Methylazoxymethanol on Rat Mesonephros and Metanephros in Organotypic Culture

Previous Serial Number: Same

Principal Investigator: Shozo Takayama, M.D. (Eleanor Roosevelt Fellow)

Other Investigators: G.L. Laqueur, M.D.; M. Spatz, M.D; and C.J. Dawe, M.D.

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional:
Other:

Project Description:

This project is a continuation of Dr. Takayama's work started in this laboratory in 1970 as an Eleanor Roosevelt Fellow. Since Dr. Takayama's return to Tokyo, portions of the work have been carried on here, but most of it is being done in Tokyo, by Dr. Takayama.

Objectives: Methylazoxymethanol (MAM) is the active aglycone moiety of the naturally-occurring carcinogen, cycasin. It was shown by Laqueur and collaborators to induce renal neoplasms in adult rats and to induce neoplasms of intestine and kidney in the offspring of female rats exposed to the carcinogen during pregnancy. It therefore can act transplacentally on renal tissues during relatively early stages of renal development. The object of this project is to determine whether MAM induces neoplastic change in embryonic mesonephros and metanephros in organ culture.

Methods Employed: Accurately staged Fischer rat embryos are aseptically removed from the uterus at 13, 14, and 15 days of gestation. Mesonephric and metanephric rudiments are excised and exposed to MAM acetate at several concentrations for 12 or 24 hours, and are then placed in gelatin sponge matrix cultures for periods up to 30 days. Living cultures are observed for toxic effects of the carcinogen, and cultures are subsequently fixed, serially sectioned, and examined histologically for evidence of changes consistent with neoplastic transformation. In other experiments, similar kidney rudiments are exposed to the carcinogen and are then transplanted to newborn syngeneic rats to see if neoplasms arise in the exposed, transplanted organs. Experiments

similar to those in organ culture are done with monolayer cultures prepared from embryonic and newborn rat kidney.

Transplacental effects of MAM acetate on the developing meso- and metanephros are looked for after injecting 20 mg/kg I.P. into female rats on the 13th day of pregnancy. One day later the embryos are removed and the mesonephros and metanephros are placed in organ culture and observed as described above.

Major Findings: Morphologic changes consistent with neoplastic transformation have not been seen in organ cultures of either mesonephros or metanephros directly exposed to MAM acetate in culture. On the other hand, the same rudimentary organs from embryos exposed to MAM acetate *in utero* have developed changes suggestive of nephroblastoma (Wilm's tumor). These cultures have given rise to several cell lines, only one of which is currently being maintained. Control cultures have thus far not given rise to continuous cell lines, but additional control cultures are presently under observation to make certain whether or not this difference occurs without exception.

Transplants of renal tissue exposed *in vitro* and then transferred to new syngeneic hosts have not given rise to tumors, but have developed into large cystic lesions in a few instances.

Significance: The findings suggest that MAM is not an ultimate carcinogen, as it has been thought to be. However, it apparently can be metabolized into an ultimate carcinogen somewhere in the adult rat, and the active product can cross the placenta to induce changes in the fetal nephric tissues that manifest themselves as neoplasia when the nephric tissues are grown in organotypic culture. It was previously shown by Laqueur et al that MAM is able to induce renal tumors in the offspring of rats given MAM during pregnancy.

The findings indicate that a carcinogenically active metabolite of MAM should be sought.

Proposed Course: Cultures of the cell line derived from mesonephros exposed to MAM and its metabolites *in utero* will be transplanted into newborn syngeneic Fischer rats to determine their tumorigenicity. Dr. Takayama will complete the culture experiments in Tokyo, and then plans to return to NCI for a few months to review the data and write a report.

Serial No. NCI 584
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Effects of Cyclic AMP on Mouse Salivary Gland Development In Vitro

Previous Serial Number: None

Principal Investigator: Clyde J. Dawe, M.D.

Other Investigators: W.D. Morgan; G. Johnson, M.D.; and I.H. Pastan, M.D.

Cooperating Units:

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional:
Other:

Project Description:

Objectives: The nucleotide adenosine-3',5'-cyclic monophosphate is known to be a mediator of control of a number of cellular processes, among the most notable being responses to hormones and release of neurotransmitter substances. Another effect of cyclic AMP, recently reported by members of this group of collaborators, (ref. below) is to decrease the motility of certain connective tissue cells (L-929) in subconfluent cultures. Further, it has been found that associated with contact inhibition of movement and growth of 3T3 cells there is a rise in endogenous cyclic AMP levels. In general, cyclic AMP appears to influence growth and/or motility of connective tissue cells in culture, but has little directly discernible effect on isolated epithelial cells under comparable conditions.

As salivary gland organogenesis has been shown to depend on tissue-specific interaction between salivary mesenchyme and salivary epithelium, it follows that substances capable of altering the biologic function of the mesenchymal component should also alter the overall developmental process. Since cyclic AMP alters motility and growth of cells from certain mesenchymal cell lines, it might be expected to alter these parameters of salivary mesenchyme and indirectly to alter salivary gland development. These experiments were designed to test this postulate.

Methods Employed: The organ culture system previously used by us in studies of polyoma virus-induced transformation in salivary glands in vitro was adapted to this study. Submaxillary salivary rudiments are excised from

13- to 15-day mouse embryos of strain C_3H/Bi, and embedded in chicken plasma clot on coverslips in Leighton tubes. Cultures are maintained in 1.0 ml Eagle's Minimal Essential Medium, with daily complete replacement of the liquid phase. The substances under test include dibutyryl cyclic AMP, alone and in combination with theophylline (a potentiator of dbcAMP); theophylline alone; epinephrine (which induces changes in endogenous cAMP); isoproteronol, a catecholamine that induces increased endogenous cAMP in heart muscle and increased DNA synthesis and hyperplasia in murine salivary glands; prostaglandin E_1, which alters endogenous cyclic AMP levels, usually upwards; insulin, both alone and in combination with prostaglandin E_1; and ethyl alcohol (control), the vehicle for prostaglandin E_1.

Cultures are carried for 18-24 hours before subjecting them to test substances renewed daily for the subsequent 5 days. Daily observations are made on the progress of development of the rudiments, and photographs of representative changes in test and control cultures are made. In some runs, daily counts of the numbers of terminal buds on the developing adenomeres are made. It is planned to make low-magnification time-lapse cinematographic recordings of selected response patterns, to be compared with similar recordings of normal gland development by the split-frame technique devised by Morgan. Histologic and histochemical studies are performed on serial sections of test and control glands at selected intervals during the 5-day exposure or at termination of the run. Whole-mount stained preparations of representative samples of test and control glands are made to supplement the sectioned material and the photographic record of living cultures.

Major Findings: Dibutyryl cyclic AMP has produced marked alterations in salivary gland development, duplicated in kind, though lesser in extent, by prostaglandin E_1 and theophylline. Insulin has shown a slight inhibitory action against the effects of prostaglandin E_1, but has not yet been tested for inhibition of the exogenous dbcAMP action.

The most striking gross effect of these substances was cystic dilatation of the proximal salivary duct systems, both in the submandibular gland and in the sublingual gland, which was usually included in the explants. Duct dilatation was generally evident after the first 24 hours of exposure to dbcAMP, and by the 3rd or 4th day was maximal. By that time most dbcAMP-exposed rudiments could be grossly seen as spheroidal, loculated sacs filled with water-clear fluid. There was a tendency for rudiments with the most pronounced cystic change to come loose from the coverslips and drift in the medium as fluid-filled sphericles.

Concomitant with duct dilatation, there was a moderate decrease in rate of duct branching and formation of new terminal buds. This effect, however, was often difficult to evaluate in living cultures because cystic change rapidly obscured the visibility of terminal parts of the adenomeres. In the dbcAMP and prostaglandin E_1-treated cultures, there frequently appeared a monolayer outgrowth of epithelium extending from the ostium of the duct where it was in contact with the coverslip. Such epithelial outgrowths also occasionally emanated from peripheral parts of the epithelial tree. Epithelial

outgrowths of similar type were less frequent and less extensive in the control cultures.

Associated with these changes in the epithelial tissue component were alterations in the mesenchyme in dbcAMP-and prostaglandin E-treated rudiments. The corona of mesenchymal outgrowth on the coverslip at the base of attachment of the rudiment was slightly smaller in diameter than in controls, and the mesenchymal cells were less compactly arranged, forming a looser meshwork than in control cultures. There was also a tendency for mesenchymal cells to be more elongated, with long, sharp-tipped bipolar cytoplasmic processes, and to be arranged on the periphery in a more distinctly radial pattern. The mesenchymal cells in treated cultures tended to contain cytoplasmic vacuoles or granules at either end of the elongated nucleus, whereas the untreated mesenchymal cells had more uniformly clear, refractile cytoplasm.

Study by histologic sections is as yet in a very preliminary stage. The lumens of the dilated duct systems appear completely empty, except for some cellular debris normally to be expected. This suggests that the secreted material presumptively responsible for the duct dilatation contains little protein and is highly water-soluble. It has not yet been determined whether the duct dilatation results from hypersecretion, duct obstruction, or a combination of the two. In fixed whole mounts the ostia of the ducts appear to be open. However, we have no information regarding the possibility that contractility of periductal smooth muscle may be impaired. Time-lapse studies may answer this question. There is also a possibility that function of the paraductal parasympathetic ganglion may be involved. Histologically the neurones of these ganglia do not appear abnormal, but they have not been examined by special neurocytologic techniques.

It is our tentative impression that in dbcAMP-treated rudiments there is precocious appearance of mucoprotein secretory activity in cells of the terminal buds of the adenomeres. Cells filled with (presumptive) mucoprotein are present in these buds, and are numerous in occasional foci. Such foci have not been seen in control rudiments, but more extensive study is required.

Quantitative evaluation of the effects of dbcAMP on proliferative activity of epithelium and mesenchyme have not been made, but it can at least be stated that mitoses in both tissues are numerous, as they are also in controls. Possibly there is an increase of mitotic index in the epithelial sheets growing out from the proximal ends of the ducts. Tripolar mitoses and anaphase figures with lagging chromosomes are quite commonly present.

Significance: There are numerous examples showing that factors influencing growth and differentiation of tissues also influence the responsiveness of these tissues to carcinogenic agents. The increased frequency of mammary cancer in mice carrying the mammary tumor virus after increased stimulation with prolactin may be cited as one. More apropos to the present investigation are our own previous studies showing that continued epithelial-mesenchymal interaction, necessary for salivary gland development and, ultimately, cytodifferentiation, is essential for ellicitation of the neoplastic response to

polyoma virus. It is therefore relevant to analyze the effects of any agent, such as cyclic AMP, that influences development and differentiation of salivary gland. If the mechanism of action of this mediating agent can be clarified, it is reasonable to presume that the cell-virus interaction leading to neoplasia will also be clarified.

When this investigation was initiated, it was not known whether cyclic AMP would have any effect on salivary gland organogenesis or cytodifferentiation. It is now apparent that cAMP does have a profound effect on organogenesis, and probably has an effect also on secretory activity of developing salivary rudiments. It was previously shown by others that catecholamines stimulate increased levels of amylase in the saliva. This stimulatory effect was shown (Bdolah and Schramm, Babad et al) to be mediated by cyclic AMP. Further, Selye found that isoproteronol, whose effects are also mediated by cAMP, induced a six-fold weight increase in rat salivary glands. Barka, Baserga, and others have found that isoproteronol stimulates a marked increase in DNA synthesis and mitotic activity in rat salivary glands. Such observations in conjunction with those made in this study suggest that cAMP may prove to be an extremely useful tool in extending our investigations of polyoma-induced salivary gland neoplasia.

Proposed Course: The present study as outlined will be carried to completion as rapidly as possible. In addition, we plan to look at the effect of cAMP on salivary tumor tissue in vitro, to see if it produces changes in differentiated activity of tumor cells. Many other studies suggest themselves, such as the administration of isoproteronol or theophylline to mice infected with polyoma virus, to see if such stimulation of cAMP activity influences the incidence of salivary gland tumors. Studies of the response of salivary rudiments to polyoma virus in the presence of added cAMP are also indicated. Further analysis of the effects of cAMP on salivary organogenesis should be done by studying the effect of cAMP on the isolated epithelial and mesenchymal components of the rudiments.

Publications:

1. Johnson, G.S., Morgan, W.D., and Pastan, I.: Regulation of cell motility by cyclic AMP. Nature 235: 54-56, 1972.

Serial No. NCI 586
1. Laboratory of Pathology
2. Comparative Oncology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: A Study of Epithelial-Mesenchymal Interactions in Relation to Mammary Carcinogenesis

Previous Serial Number: None

Principal Investigator: Teruyo Sakakura, M.D. (Visiting Associate)

Other Investigators: C.J. Dawe, M.D.

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional:
Other:

Project Description:

Objectives: In all organs with epithelial parenchyma excepting the crystalline lens, the epithelium is supported by and continuously interacts with a stroma of mesenchymal cells. Transport to epithelium of nutrition, gases, endocrine products, water, electrolytes, and even of systemically administered carcinogenic agents must go through the stroma, lying between the vascular supply and the epithelium. During embryogenesis, the interaction between stroma and epithelium has been shown to be essential for normal organogenesis and, ultimately, cytodifferentiation. Even in the adult a state of dependency of epithelium on its specific type of stroma exists, as manifested for example by the inability of hair follicles to persist and produce hair if the follicle papilla is lost or destroyed.

In view of the supportive and regulatory roles of stroma during normal organogenesis and organostasis, it seems probable that stroma mediates or influences critical events during carcinogenesis. That this is so in mammary carcinogenesis has been demonstrated by DeOme and coworkers who have shown that mouse mammary epithelium in the absence of mammary fat pad fails to grow or to form hyperplastic nodules in response to mammary tumor virus. In the absence of hyperplastic nodules, mammary adenocarcinomas in turn fail to develop. Hence, through interference with the epithelial-mesenchymal interaction system, MTV-induced mammary neoplasms can be indirectly prevented, though at the cost of preventing normal development and function of the gland.

In the salivary gland/polyoma virus-induced tumor system, a similar situation exists. When salivary gland epithelium is isolated from its proper mesenchyme or is recombined with "strange" mesenchyme, it fails to develop into normal salivary gland or to become neoplastic in response to polyoma virus.

The objective of this project is to determine whether and in what way mammary carcinogenesis can be influenced by exposing only one or the other component (epithelium or mesenchyme) to a carcinogenic stimulus that is highly effective when applied to both components interacting normally.

Methods Employed: The experimental tumor-induction system to be used is X-irradiation-induced mammary neoplasia in inbred Sprague-Dawley rats. Females of this strain develop mammary neoplasms following total body irradiation by 400r, at an incidence of 60-80 percent within one year.

For technical reasons, it will be necessary to induce mammary tumors by irradiating the animals in utero, at days 16-17 of gestation. Therefore a preliminary experiment is being done to determine the incidence of mammary tumors induced by 400r administered at gestation days 16-17. This will provide necessary baseline data.

The analytical phase of the experiment calls for excision of the mammary gland rudiments from 16-17 day embryos, followed by trypsin separation of these rudiments into their epithelial and mesenchymal components. In each experimental run, half of the separated components will be irradiated with 400r in vitro, and the other half will be untreated. Irradiated epithelium will then be recombined with non-irradiated mesenchyme, and irradiated mesenchyme will be recombined with non-irradiated epithelium. These recombinations will be allowed to become reorganized in culture dishes for 24 hours and will then be transplanted subcutaneously to syngeneic newborn rats. Controls in which neither component is irradiated and in which both components are irradiated will be similarly handled. All groups of recipients will then be observed for appearance of tumors at the site of transplant. When tumors reach a size of 2 cm in diameter, the hosts will be autopsied and histologic study of the tumors will be done. Tumors induced in the recombinations will be compared with those induced in intact rudiments, those induced by irradiation of whole embryos in utero, and those induced by total-body radiation of weanling females. Incidence rates and histological features of the neoplasms in the various groups will be compared.

Major Findings: Most of this year has been required to work out the techniques and to determine feasibility of certain aspects of the experiment. The experiment will be continued at the Aichi Cancer Research Institute after the return of the Principal Investigator to Nagoya near the end of June.

It has been found practicable to transplant intact 16-day mammary rudiments subcutaneously to the ears of female mice. Both male and female rudiments undergo fairly extensive development, though not to normal size under these conditions. They also show secretory activity in female recipients

during lactation after pregnancy. Since the male rudiments behave the same as female rudiments when placed in female hosts, it will be possible to use male as well as female donors in the later work.

It has also been found that isolated epithelium from 16-day rudiments develops into a rather extensive branching, typical mammary duct system when transplanted into the cleared mammary fat pad of 3 to 4-week old female rats. This observation had not previously been made or reported, but was perhaps to be expected in view of the demonstration by DeOme that fragments of adult mammary gland are able to undergo extensive growth and normal duct formation when transplanted into the cleared mammary fat pad. In the present work, it was also found that isolated mammary rudiment epithelium is incapable of growth and duct formation when transplanted outside the mammary fat pad. These findings make it possible to carry out the experimental plan in an additional way that may have advantages. That is, female rats of 3-4 weeks age can be irradiated after clearing a fat pad. Unirradiated rudimentary epithelium can then be implanted in the cleared, irradiated fat pad and the incidence of tumors arising in glands in those fat pads determined. Reciprocally, irradiated epithelium can be implanted in non-irradiated, cleared fat pads, and the results observed. The advantage of this method is that isolated epithelium transplanted to adult fat pad undergoes more extensive development than when it is recombined with fetal mammary mesenchyme and transplanted subcutaneously.

Another exploratory experiment has yielded information potentially useful to the main experimental plan. It was found that rudimentary mammary epithelium recombined with rudimentary salivary gland mesenchyme undergoes atypical development when the combination is transplanted subcutaneously to a newborn host. The resulting gland has a duct pattern more nearly like that of salivary gland. Similar results had previously been reported by Kratochwil in experiments done entirely in culture, although development is more extensive in the experiments in vivo. This information makes it possible to determine whether combinations of mammary epithelium with heterotypic mesenchyme suppresses or enhances the neoplastic response to irradiation.

Significance: This will depend on the results, which are not yet available. The design of the experiment is such that it will enable one to determine whether the carcinogenic action of X-irradiation on mammary gland is directed through only one or the other of the two tissue components of the gland, or both. At present it is known from previous experiments that the carcinogenic effect is directly exerted on the mammary gland, and not upon some other organ (such as an endocrine gland) which indirectly influences the mammary gland. The present experiment offers a still higher degree of resolution of the target involved in X-ray induced mammary neoplasms. Two possibilities among many are: 1) that the critical event is in epithelium, as a result of radiation damage to DNA; 2) that radiation acts on mammary mesenchyme to release a latent virus, which then transforms the epithelium.

Proposed Course: The experiments will be continued as planned, with some modifications as experience requires. A colony of inbred Sprague-Dawley rats will be started at the Aichi Institute from breeders supplied by NIH, so that the work can be carried on in Japan.

Serial No. NCI 527
1. Laboratory of Pathology
2. Cytopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunocytology and Physiopathology of Cancer

Previous Serial Number: Same

Principal Investigator: Richard A. Malmgren, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total: 2
Professional: 2
Other: 0

Project Description:

Objectives: 1. To apply the fluorescent antibody techniques and other immunologic methods to the study of tumor protein and antigens. 2. To study the immunologic and other factors involved in carcinogenesis.

Methods Employed: 1. The fluorescent antibody technique has been used to study various antigens associated with neoplastic cells. This requires the preparation of antisera to the appropriate antigen and tagging the antibody with fluorescein isothyocyanate. The antisera thus prepared are applied to the cells under study, either tissue imprints, tissue culture cells or frozen sections and examination of these cells in an ultraviolet microscope. Studies were carried out using these methods on human tumor material in collaboration with Drs. Wells, McIntire and Soloway. No significant findings resulted from these studies.

Proposed Course: This project is being phased out with the retirement of the Principal Investigator.

Publications:

1. Malmgren, R.A., and Morton, D.L.: Viral and immunologic studies of human neoplasms. In Ioachin, H.L. (Ed.): Pathobiology Annual. New York, Appleton-Century-Crofts, 1971, pp. 63-81.

2. Morton, D.L., Eilber, F.R., and Malmgren, R.A.: Immune factors in human cancer: malignant melanomas, skeletal and soft tissue sarcomas. Progr. Exp. Tumor Res. 14: 25-42, 1971.

Serial No. NCI 852
1. Laboratory of Pathology
2. Cytopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Exfoliative cytology applied to human diagnostic problems and research problems

Previous Serial Number: Same

Principal Investigator: Richard A. Malmgren, M.D.

Other Investigator: Elizabeth W. Chu, M.D.

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total: 12
Professional: 10
Other: 2 *

*Kenneth Nakahara joined this laboratory in May 1971 as a biologist processing material received for chromosomal analysis.
*William Crafford accepted a temporary position to work in the area of cytogenetics from January 1928 - August 30, 1972.

Project Description:

Objectives:
The paramount responsibility of the Cytopathologic Section is to provide the Clinical Center with an accurate and complete exfoliative cytology as well as diagnostic cytogenetic services. In addition the Cytopathology Section collaborates in various clinical research projects evaluating cancer therapy, the hormonal status of patients when requested, the course and natural history of the cancer lesion, and the anatomical and physiological changes in the human body associated with various pathologic conditions. The accomplishment of these objectives requires the continual improvement of techniques and the origination of new cytologic methods in the laboratory.

The specific objectives include: (1) Development of methods for the automation of cytology. (2) Application of the automation methods being used to study basic normal and malignant cell characteristics. (3) Evaluation methods for individual chromosomal identification. (4) Work to improve methods for cancer diagnosis. (5) Evaluate aspiration biopsy material to develop criteria for cytologic diagnosis of cancer. Material to be obtained from various sites. (6) Evaluate the cytologic characteristics of normal cellular composition of ileal loop urine, where recurrent cancer may be recognized

2. The second major contribution of the Cytopathology Section is to the research programs of NIH which require cytologic and/or cytogenetic methods.

3. Routine cytogenetic methods are now established so that the Cytopathology Section can provide this service for clinical studies. Special staining methods in assessing chromosomal changes are being initiated.

4. The value of exfoliative cytology in aspiration material is being assessed.

5. Cytologic evaluation of urine from ileal loop patients has been useful in detecting new and/or recurrent tumors.

6. Cytogenetic studies have been useful in establishing definitive diagnosis in patients with endocrine disorders as well as patients with unusual mosaicism.

Significance:

1. The diagnostic value of exfoliative cytology in the clinical evaluation of cancer and its early detection is well established in the Clinical Center and throughout the country. Unsuspected in situ carcinoma of the cervix has been detected in Clinical Center patients by routine examination of vaginal and cervical smears. Exfoliative cytology has often been the only tissue evidence of cancer in some patients prior to treatment or death.

2. The need for more cytogenetic studies on a service base as well as research basis has been in demand for some time and now its application is expanding.

Proposed Course:

The continuation of a complete and adequate cytopathology service for the Staff of the Clinical Center is our primary goal. Continued collaboration with the Clinical Center Staff in various research projects and the development of new and improved techniques within the laboratory are an important part of the future program. Special emphasis will be placed on cytogenetic studies, evaluation of new cancer detection tests, special staining to assist in chromosomal and cytologic characterizations and morphological features of cells seen in needle-aspirated materials. A major effort will be placed upon studies to automate cytology.

Work in Progress:

1. In collaboration with Drs. Chu, Bamford* and Gilfillan* human fibroblasts are being exposed to SV40 virus and the % transformed determined. An analysis will be made to determine if any correlation exists between the % transformed and whether or not the patient has a malignancy.

2. To study and compare the X-chromosomes in women with and without neoplastic diseases. Collaborative work with New England Medical Center.

* New England Medical Center

as it arises. (7) Conduct experimental studies of possible etiologic factors for carcinoma of the uterine cervix.

Methods Employed:

The standard Papanicolaou staining procedure is applied to preparation of various human specimens. These specimens are then screened and evaluated for morphologic changes in the cellular elements. Special procedures involving the use of fluorescent microscopy, millipore filtration, and various special staining and centrifuge techniques are required in many instances. Cultures of patient peripheral blood, bone marrow and tissues are prepared and the chromosomes analyzed. Coulter counter and cell separation methods are used.

Patient Material:

The figures compiled below are for the last twelve months.

March 1971 - February 1971

Type of Material Examined	Accessions	Smears
Vaginal and Cervical	1,206	2,107
Other (Ascitic, pleural, gastric, prostatic, urine, bronchial washings, sputa, spinal fluids, sex chromatin, wound washings, breast, oral, aspirations, chromosome analysis, chromasome tissue analysis, and spinal procedures	3,926	11,877
Total	5,132	13,984

Institutes	Accessions	Smears
NCI	3,616	9,771
NHLI	278	712
NIAMD	264	548
NIAID	194	595
NINDS	157	377
NIMH	69	137
NIDR	26	81
NEI	27	51
NICH	419	1,293
Referred	66	375
CC (EHS)	16	44
Total	5,132	13,984

Major Findings:

1. The major contribution of the Cytopathology Section to the diagnostic facilities of the Clinical Staff has been the early diagnosis of neoplasms of the cervix and lung and metastatic lesions of various sites not previously diagnosed. In several instances, exfoliative cytology provided the only tissue confirmation of neoplastic disease.

3. In collaboration with Dr. Aptekar, urine specimens at regular intervals from patients being treated with high dosages of cytoxan, methotrexate and other chemotherapeutic agents are studied to ascertain any damages on the urinary tract.

(This report prepared by Dr. Elizabeth W. Chu)

Serial No. NCI 854
1. Laboratory of Pathology
2. Cytopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Experimental studies on carcinogenesis, endocrine factors and cytogenetic characters

Previous Serial Number: Same

Principal Investigator: Elizabeth W. Chu, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total: 1
Professional: 1
Other: None

Project Description

Objectives:

1. To study the pathogenesis of lesions of rodents after treatment with chemical and biological agents and to study the influence of these agents on the development of neoplasms and metastases.

2. To study the possible relationship of X-chromosomes and neoplastic diseases in women.

3. To study the effects of Enovid in rodents.

4. To study the effects of Trichomonads in rodents.

Methods Employed:

1. Hamsters were fed with a mixture of ground purina chow containing:
   (a) 0.05% DMBA tap water to drink
   (b) 0.05% DMBA 5% alcohol to drink
   (c) 0.05% DMBA 10% alcohol to drink
   (d) 0.05% DMBA 20% alcohol to drink
   (e) 0.05% DMBA 30% alcohol to drink

Collaborator: Dr. R. A. Malmgren

2. Peripheral blood lymphocytes and biopsies of skin from patients with and without neoplastic diseases are cultured for chromosomal analysis. Tritiated thymidine is used to label the X-chromosomes for positive identification; and/or special staining to identify the X-chromosomes will be applied. The arm lengths of the X-chromosomes of cancerous patients will be compared with those of non-cancer patients. (In collaboration with Dr. Sophia Bamford of NEMC).

3. Three groups of 25 mice in each group are fed with processed Enovid pellets in this study. The Enovid powder is mixed with Gilford's powdered chow and pressed into pellets containing (a) 5 micrograin per gm. of chow, (b) 10 micrograin per gm. of chow and (c) 20 micrograin per gm. of chow.

A fourth group of 20 mice served as controls are given Gilford's chow pellets.

Vaginal smears will be examined at monthly intervals for early detection of neoplastic changes. (Collaborator: Dr. R. A. Malmgren)

4. To study the effects of Trichomonads three groups of mice are set up as follows:

(a) 20 mice will be given 10 micrograms of Enovid per day and 0.1 ml. of Trichomonads in culture medium per vagina at weekly intervals.

(b) 20 mice same as above but given weekly culture medium without the organisms.

(c) 20 mice on daily Enovid only to serve as controls.

Similar groups of Syrian hamsters have been set up but using doses of Enovid double that of mice

Vaginal smears will be examined at weekly intervals for early detection of neoplastic changes. (Collaborator: Dr. R. A. Malmgren).

5. Cytogenetic studies on established breast cancer cell lines. (In collaboration with Dr. Bassin and Dr. Plata).

6. Cytogenetic studies on chimeric marmosets. (In collaboration with Dr. Rabson).

Major Findings:

1. The incidence rate of DMBA (dimethylbenzanthracene) induced gastrointestinal cancer is influenced by the addition of alcohol. The first part of the experiment showed that the incidence rate of the animals using 5% and 10% alcohols as drinking water were higher than those using 20% alcohol (100% versus 50%) while 30% alcohol was incompatible with life. (Collaborator: Dr. R. A. Malmgren).

2. Several cases have been reported by the New England group of investigators showing a definite difference in the measurements of the sex chromosomes where the X-chromosomes were larger in the tumor bearing females than the non-cancer bearing women.

3. The possible cancer promoting effect on the mice that had been fed with Enovid was reported. The present study also revealed a very low incidence but thought provoking cancerous growth of the uterine cervix. There were also indications of increased incidences of mammary tumor in the Balb/c mice used. Vaginal smears were not helpful in the cases of mice. Data is presently being assembled for detailed analysis.

4. Preliminary studies on the established cell lines on the cancer of breast indicative of markers being present in large percentages of cells examined but the markers are not pathognomonic of this particular cancer.

5. Cytogenetic studies on a cell line derived originally from a lymph node of a marmoset revealed both male and female cells in a 10 months' period.

Significance:

1. The promoting effect of alcohol in the induction of cancer by hydrocarbons is noteworthy, especially in the case of DMBA where the neoplastic state is 50% more when 5% or 10% alcohol was used as drinking water as compared to those using 20% alcoholic drinking water. This has practical implications in the case of humans since a large quantity of alcohol is being consumed by many people.

Final analysis of the results is presently underway.

2. Should it be ascertained that the X-chromosomes do measure differently between patients with and without cancers the technique could be applied as a screening method to sort out cancer-prone females from the general group of women. Due to factors beyond our control numerous cultures were contaminated. The experiment is being resumed and on new materials.

3. Since millions of American women are put on daily Enovid pills, a definite cancer-promoting effect of this drug needs to be further substantiated and investigated more thoroughly.

4. Cytogenetic analysis assists in ascertaining a particular established cell line; and the early recognition of an inadvertent contamination by another cell line.

Proposed Course:

1. To study sex-chromosome differences between tumor-bearing and non-tumor bearing females.

2. Continue to study the effects of chemotherapy on chromosomal changes.

3. Continue to study the effects of Enovid and Enovid in conjunction with Trichomonads.

4. To study the promoting effect of alcohol in the induction of stomach cancer, with special interest in the time factor.

5. Continue to study chimerism in marmosets.

Publications:

1. Chu, E.W. and Rabson, A.S.: Chimerism in lymphoid cell culture line derived from lymph node of marmoset infected with Herpesvirus Saimiri. J. Nat. Cancer Inst. 48: 771-773, March 1972.

2. Malmgren, R.A., Soloway, M.S., Chu, E.W., DelVecchio, P.R. and Ketcham, A.S.: Cytology of ileal conduit urine. Acta Cytol. 15: 506-509, 1971.

Serial No. NCI 569
1. Laboratory of Pathology
2. Hematopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Control of Lymphoma Development in Mice After Dilantin Treatment

Previous Serial Number: Same

Principal Investigator: Gerhard R.F. Krueger, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/12
Professional: 1/12
Other: 0

Project Description:

See annual reports 1969/1970 and 1970/1971; the experiment is terminated. One article was published and another submitted for publication during the last fiscal year.

Publications:

1. Krueger, G.R.F., and Harris, D.: Is phenytoin carcinogenic? (Letter to the Editor). Lancet 1: 323, 1972.

Serial No. NCI 572
1. Laboratory of Pathology
2. Hematopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunological Lymphoma Development

Previous Serial Number: Same

Principal Investigator: Gerhard R.F. Krueger, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional: 1/2
Other:

Project Description:

Objectives: Investigating different experimental models simulating the effects of transplantation and immunosuppression in mice. The ultimate task is to elucidate the cause of lymphoma development in transplant recipients with immunosuppressive therapy, or in congenital immune deficiency syndromes.

Methods Employed: Experiments in mice are done using continuous antigenic stimulation together with immunosuppression for lymphoma induction. As antigens serve a variety of non-oncogenic viruses, bacteria, or proteins, immunosuppression is performed with azathioprine, methotrexate, or triethylene melamine. Lymphomas are subsequently further investigated by light microscopy, electron microscopy, tissue culture methods, and immunofluorescence studies for the demonstration of tumor antigens and theta antigens.

Major Findings: Malignant transplantable (not cell-free) lymphomas were induced in 20-66% of experimental mice (controls 0-12% when only immunosuppressed). Tumor cells do not cross react when checked by isoantisera; they are theta antigen positive, negative for immunoglobulin surface receptors, and grow in tissue culture as established lymphoblastic cell lines. The original tumor and early tissue culture lines are essentially free of virus particles. Late tissue culture sublines show C- and A-type particles (above the 50th subculture).

Significance: The results have an immediate bearing on human immunopathology and lymphoma development and may influence treatment of immunologic and neoplastic disorders in the future. It is felt that reticulum

cell foci in atrophic lymphoreticular tissues as described above may constitute "germ centers" for later lymphoma development.

Proposed Course: To be continued by more extensive use of electron microscopy and determinations of the immunological reactivity of the individual animal. Electron microscopic studies to be supplemented by immunological methods to determine spontaneous trends of differentiation in lymphoma cell lines.

Publications:

1. Krueger, G.R.F.: Impaired immunologic reactivity as pacemaker for lymphoma development. Verh. Deutsch Ges. Path. 55: 200-205, 1971.

2. Krueger, G.R.F., and Heine, U.I.: Morphogenesis of two immunologically induced mouse lymphomas. Cancer Res. 32: 573-582, 1972.

3. Krueger, G.R.F., and O'Conor, G.T.: Epidemiologic and immunologic considerations on the pathogenesis of Burkitt's tumor. In Grundmann, E. (Ed.): Recent Results in Cancer Research. (in press).

4. Krueger, G.R.F.: Chronic immunosuppression and lymphomagenesis in man and mice. Nat. Cancer Inst. Monograph (in press).

Serial No. NCI 575
1. Laboratory of Pathology
2. Hematopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Histopathologic Study of Graft-Versus-Host Reaction in Human and Animal Bone Marrow Allograft Recipients

Previous Serial Number: Same

Principal Investigator: Gerhard R.F. Krueger, M.D.

Other Investigators: R.G. Graw, Jr., M.D.; G.P. Herzig, M.D.; C.B. Merritt, M.D.; and G.N. Rogentine, M.D.

Cooperating Units: Human Tumor Cell Biology Branch and Leukemia Service, Medicine Branch; Immunology Branch, National Cancer Institute, National Institutes of Health

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

Objectives: To investigate the effect of HL-A typing and variable chemotherapeutic measures on the development of graft-versus-host disease. To define graft-versus-host disease morphologically by exacting differential diagnosis. To relate the extent of morphologic changes in graft-versus-host disease to the prognosis of the patient.

Methods Employed: Histopathological methods on materials from human and animal allograft recipients (dogs and monkeys). The animal experiments include extensive controls with transplantation of autologous bone marrow, chemotherapy, administration of antilymphocyte sera, and x-irradiation.

Major Findings: 1. The diagnosis of graft-versus-host is possible histologically, although often difficult to separate from lesions secondary to infection.

2. Graft-versus-host may occur in genotypically matched individuals indicating that loci other than of the HL-A system may be important in the pathogenesis of graft-versus-host.

3. Graft-versus-host reaction may occur despite lack of evidence for graft take indicating that "takes" of the various cell lines transplanted cannot be correlated.

4. Graft-versus-host reaction may occur after transfusion of viable blood cells.

Significance: These results have an immediate influence on future studies to select donor and recipient, and to evaluate the effect of treatment.

Proposed Course: To be continued as indicated; the investigation of human materials is done on a "service basis" involving a large number of investigators from different laboratories.

Publications:

1. Graw, R.G., Jr., Yankee, R.A., Leventhal, B.G., Rogentine, G.N., Herzig, G.P., Halterman, R.H., Merritt, C.B., Carolla, R.L., Alvegörd, T.A., Bull, J.M., McGinnis, M.H., and Krueger, G.R.F.: Bone marrow transplantation in acute leukemia employing cyclophosphamide. Exp. Hematol. (in press).

2. Halterman, R.G., Graw, R.G., Jr., Leventhal, B.G., Johnson, G., House, S., and Krueger, G.R.F.: Reactive lymphocyte blastogenesis during graft-versus-host reaction. Transplantation (in press).

Serial No. NCI 576
1. Laboratory of Pathology
2. Hematopathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Histopathologic Study of Side Effects of Cancer Chemotherapy with Special Attention to the Immunosuppressive Effect

Previous Serial Number: Same

Principal Investigator: Gerhard R.F. Krueger, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

Discontinued as separate serial number; work continued as part of projects with serial numbers NCI 572 and NCI 575.

Serial No. NCI 517
1. Laboratory of Pathology
2. Pathological Technology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 - June 30, 1972

Part A:

Project Title: Pathological Technology Section

Principal Investigator: Miss Betty J. Sanders

Other Investigators:

Cooperating Units:

Man Years (July 1, 1971 - June 30, 1972)
Total: 15
Professional: 3
Other: 12

1. Project Description:

Stained tissue sections are the fundamental basis of all clinical and experimental studies of cancer. The Section prepares histological sections for the investigators of the National Cancer Institute. It makes available all the established routine and special stains and in addition develops and provides the current experimental methods of tissue preparation such as enzyme stains and specific histological stains.

July 1, 1971 - June 30, 1972	
Number of investigators	101
Number of pieces of tissue	45,440
Number of bottles of tissue	9,225
Number of blocks cut	31,790
Number of blocks cut serially	838
Number of frozen blocks cut	54
Number of blocks recut	983
Number of slides stained H & E	45,966
Number of slides stained special	5,982
Number of unstained slides	6,246
Contract Work - Baker Histology Laboratory	
Number of bottles of tissue	5,562
Number of pieces of tissue	40,832
Number of slides stained H & E	22,390
Contract Work - Hazleton Laboratories, Inc. (contract expired August 1971)	
Number of bottles of tissue	699
Number of pieces of tissue	6,937
Number of slides stained H & E	4,323

2. Approximately 900 - 1000 hours have been on compiling, typing and proofing a manual titled Animal Histology Procedures which is now in press and will be for sale at the Government Printing Office by September 1972.

3. Two employees are in the 2nd semester of the NIH course "Human Biology." This is composed of 80 hours of lectures in a 20 week period. A third employee is registered in a course of Biology at Montgomery College and will successfully complete the second semester in May 1972.

4. Renovation of the laboratory with a loss of 2895 square feet of space was completed December 20, 1971 and the move was completed January 3, 1972.

5. The contract with Baker Histology for 60,000 microscopic slides is in the 6th month with fewer than 16,000 slides completed. 30,000 should have been completed by this date. The slides are of poorer quality than those produced in the past by that laboratory.

The block storage contract was renewed in December.

Serial No. NCI 853
1. Laboratory of Pathology
2. Surgical Pathology and Postmortem Sections
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Surgical Pathology and Postmortem Sections, together with the Cytopathology Section, Ultrastructural Pathology Section and Hematopathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all Institutes in those investigations which involve the use and study of human pathological material. The staff of the Surgical Pathology and Postmortem Sections also carries on independent and collaborative research.

Staff:

Dr. Gregory T. O'Conor, Head, Surgical Pathology Section
Dr. Ralph D. Powell, Jr., Surgical Pathology Section
Dr. Louis B. Thomas, Chief, Laboratory of Pathology and Head, Postmortem Section
\+ Dr. Hugh Bonner, Resident
\+ Dr. L. Max Buja, Resident
\+ Dr. Joseph M. Davie, Resident
\+ Dr. Elaine Jaffe, Resident
\+ Dr. Howard Marton, Resident
\+ Dr. Brian R. Mullin, Resident
* Dr. Leon Sokoloff, Laboratory of Pathology and Histochemistry, National Institute of Arthritis and Metabolic Diseases
* Dr. George Garrington, Deputy Director of Intramural Research, National Institute of Dental Research
* Dr. William C. Roberts, Chief, Laboratory of Pathology, National Heart and Lung Institute
* Dr. Marius Valsamis, Chief, Diagnostic Neuropathology, Bronx Municipal Hospital, and Assistant Professor of Pathology (Neuropathology), Albert Einstein College of Medicine

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\+ - These physicians are full time Residents in the Laboratory of Pathology.
* - These Associate Pathologists spend part time in the activities of the Surgical Pathology and Postmortem Sections.

Project Description:

Objectives: The objectives of the Surgical Pathology and Postmortem Sections are: (1) to provide diagnostic services in pathologic anatomy; (2) to collaborate with National Institutes of Health investigators in research involving use and study of human material; (3) to conduct a residency training program in pathologic anatomy, and (4) to carry out independent research.

1. The service functions of the Sections during the period April 1971 through March 1971 included:

(a) 248 autopsy examinations. Included in this report is a table which shows the number of autopsies performed for each Institute. The residents perform nearly all of the postmortem dissections under supervision of the staff. A gross review is held by the staff and residents each morning at 8:30. The residents review the microscopic slides of each autopsy with one of the staff before completing the autopsy protocol.

The staff assists the residents in preparing for the numerous clinical conferences in which the Sections participate.

(b) 6229 specimens were accessioned in the Surgical Pathology Section. A complete listing of these specimens, by Institute, is given in the accompanying table. These specimens are initially examined and described by a resident and their reports are checked by the staff. Associate pathologists from other laboratories or sections are frequently consulted about problems in diagnosis in surgical pathology and/or assume responsibility for handling certain tissue removed for specific research purposes.

(c) Histological preparation and special procedures:

These functions are carried out under the direction of Miss Mary C. Bowling and a complete listing of specimens processed by Institutes is given in the accompanying table.

(d) Photographic Services of the Department

A photographic record is made of a large amount of the pathological material which is handled and studied in the department. The photography is done by the staff and residents with the technical assistance of Mr. Kenzie Edwards who also maintains the equipment and carries out much of the photographic processing. Gross photographs and photomicrographs of the pathologic material are provided to the clinical staff on request, are used extensively for conferences and seminars and many are prepared for publication.

During the past year Mr. Edwards took 2,810 color slides, 132 prints, 8 black and white slides, 2138 black and white negatives, and 193 color rolls of 35 mm. film.

Serial No. NCI 853

REPORT OF POSTMORTEM SECTION

APRIL 1, 1971 through MARCH 31, 1972

INSTITUTE

	NCI	NHLI	NIAMD	NIAID	NINDS	NIMH	NINDR	NEI	NICHD	REF.	TOTAL
Autopsies performed	110	28	8	6	9	0	0	0	3	1	165
Autopsies submitted	54	17	5	0	0	0	0	0	0	7	83
Total autopsies	164	45	13	6	9	0	0	0	3	8	248
Blocks of tissue cut	5152	1254	466	373	391	0	0	0	69	50	7755
Blocks of tissue recut	855	180	123	55	103	0	0	0	11	19	1346
Blocks serialled	0	0	2	0	0	0	0	0	0	0	2
Total blocks cut	6007	1434	591	428	494	0	0	0	80	69	9103
Slides made	7945	1919	983	605	756	0	0	0	103	144	12455
Slides H&E stained	6009	1249	628	416	458	0	0	0	71	212	9043
Slides special stained	1378	480	128	131	223	0	0	0	14	69	2423
Total slides stained	7387	1729	756	547	681	0	0	0	85	281	11466
Frozen sections	45	29	3	0	1	0	0	0	0	0	78

CONTRACT: 67 autopsies sent
4619 H&E stained slides returned

Serial No. NCI 853

REPORT OF SURGICAL PATHOLOGY SECTION — APRIL 1, 1971 through MARCH 31, 1972

INSTITUTE

	NCI	NHLI	NIAMD	NIAID	NINDS	NIMH	NIDR	NEI	NICHD	REF.	TOTAL
Specimens accessioned	4570	336	348	216	111	11	506	13	72	46	6229
Blocks of tissue cut	8504	490	638	294	331	31	14	16	197	65	10580
Blocks of tissue recut	592	63	152	56	71	1	3	6	17	6	967
Total blocks cut	9096	553	790	350	402	32	17	22	214	71	11547
Slides made	29501	1755	3365	1619	1259	104	57	78	802	269	33809
Slides stained H&E	17319	999	1857	871	493	56	510	34	355	184	22678
Slides stained special	5392	263	391	340	273	6	17	21	130	31	6864
Total slides stained	22711	1262	2248	1211	766	62	527	55	485	215	29542
Frozen sections	215	36	52	7	12	4	2	0	4	0	332

962

Total Surgical and Postmortem Pathology Section Report:

Number of blocks cut ---------- 2065
Number of slides made ---------- 51264
Number of slides stained H&E ---------- 31721
Number of slides stained special 9287

A large proportion of the photomicrography is done under the direction of Mr. Ralph Isenburg who provides professional assistance and facilities for the entire staff of the Laboratory of Pathology.

2. The collaborative activities of the staff and residents in the Surgical Pathology and Postmortem Sections are reflected in the summary of research activities listed below :

(a) Distribution of human organs and tissues

At present there are approximately 30 scientists in the various laboratories at the National Institutes of Health who have requested particular tissues from surgical and postmortem services. On many occasions it is possible to furnish these investigators with fresh human material. A list of these investigators is kept by the secretarial staff and telephone calls are made to the various investigators whenever surgical or postmortem tissues are available.

(b) Conferences:

The staff and residents take an active part in numerous Clinical Center and Institute conferences. In addition, there are several conferences regularly held in the department, including a Surgical Pathology Conference, Neuropathology Conference, Joint Pathologists Conference, Staging Conference, and Gross Autopsy Conference.

A. Dr. Gregory T. O'Conor:

1. Studies of human and Simian herpes viruses. The oncogenicity and virus cell relationships of these viruses are being investigated in vitro and in vivo. In collaboration with Dr. Alan Rabson, Dr. Ruth Kirschstein, DBS, and Dr. Nubia Munoz, IARC.

2. Electron microscopy study of selected tumor and tumor cell lines. These studies involve screening for viruses and a search for specific morphological changes related to the presence of viral genome.

3. Electron microscopy of selected specimens from the surgical pathology service. The objective is to improve the accuracy of diagnoses in surgical pathology by defining ultrastructural characteristics of specific tissues.

4. Electron microscopy of virus induced renal tumor in mice. In collaboration with Dr. George Meyer, Surgery Branch.

5. Electron microscopy of penile carcinoma biopsies. In collaboration with Dr. Kiyawalsi, Department of Surgery, Makerere Medical School, Kampala.

6. Radiation therapy of lymphosarcoma. In collaboration with the Radiation Branch.

7. Geographical variations in Hodgkin's disease, Cali, Colombia and Connecticut. In collaboration with the Biometry Branch.

8. Studies in American patients with Burkitt's tumor. With VLL.

9. Epidemiological studies of Hodgkin's disease. In collaboration with the Biometry Branch.

10. Review of cancer of the oral cavity and respiratory tract in Uganda. This study is for a proposed textbook under the editorship of Dr. Templeton at the University Medical College in Kampala.

11. Hodgkin's disease in Connecticut: Histology and age distribution. In collaboration with Biometry Branch and Connecticut Cancer Registry. Will be submitted to JNCI as part of Proceedings of Symposium, Palo Alto, California.

12. Other activities:

(a) Chairman, Commission on Epidemiology of UICC.

Responsibility includes coordination of the research program of three committees and the planning of and participation in their meetings. I am actively participating in the investigations in progress by two of these committees, i.e. Childhood Cancer and Hodgkin's disease.

(b) Member, Executive Committee - UICC.

(c) Member, U.S. National Committee - UICC.

(d) Member, Breast Cancer Task Force, NCI.
Chairman, Subcommittee on Epidemiology.

(e) Member, Committee on Geographic Pathology,
International Academy of Pathology

(f) Head, Collaborating Center for International Reference
Center on Classification of Neoplasms of the Hematopoietic
System

(g) Member, N.Y. State Cancer Control Bureau
Scientific Advisory Group

(h) Member, Program Advisory Committee for Ali India
Institute of Medical Sciences

(i) Chairman, Planning Session - Objective 5, Approach 6, National Cancer Plan

(j) Consultant - International Agency for Research on Cancer

(k) Consultant - Pathology Panel, Lymphoma Task Force

(l) Consultant - Sero-epidemiology Contract Review Committee, NCI

(m) Consultant - USA Committee of International Societies of Pathology, NRC and Project Officer for Contract to support WHO International Reference Centers

Publications:

1. Rabson, A.S., O'Conor, G.T., Lorenz, D.E., Kirschstein, R.L., Legallais, F.Y., and Tralka, T.S.: Lymphoid cell culture line derived from lymph node of marmoset infected with Herpesvirus saimiri - A preliminary report. J. Nat. Cancer Inst. 46: 1099-1109, 1971.

2. Primack, A., Wilson, J., O'Conor, G.T., Engelman, K., Hull, E., and Canellos, G.P.: Hepatocellular carcinoma with the carcinoid syndrome. Cancer 27: 1182-1189, 1971.

3. O'Conor, G.T.: Relationship of classification to epidemiology of malignant lymphomas. In Clark, R.L., Cumley, R.W., McCay, Joan E., and Copeland, M.M. (Ed.): Oncology 1970 (Proc. of the Tenth International Cancer Congress held in Houston, Texas). Chicago, Year Book Medical Publishers, Inc., 1970, Vol. 5, pp. 291-299.

4. Sizaret, P., O'Conor, G.T., Beaumont, R., and Laval, M.: Serum protein patterns in mice following primary and challenge infection with Plasmodium berghei. Zeitschrift fur Tropenmedizin und Parasitologie 22: 260-270, 1971.

5. Correa, P., and O'Conor, G.T.: Epidemiologic patterns of Hodgkin's disease. Int. J. Cancer 8: 192-201, 1971.

6. O'Conor, G.T., Tralka, T.S., Henson, E., and Vogel, C.L.: Ultrastructural survey of primary liver cell carcinomas from Uganda. J. Nat. Cancer Inst. 48: 587-603, 1972.

7. Levine, P.H., O'Conor, G.T., and Berard, C.W.: Antibodies to Epstein-Barr virus (EBV) in American patients with Burkitt's lymphoma. Cancer (In press)

8. Stevens, D.A., O'Conor, G.T., Levine, P.H., and Rosen, R.B.: Simultaneous onset of acute leukemia with "Burkitt's lymphoma cells" and Burkitt's lymphoma in American siblings. Ann. Intern. Med. (In press)

9. Krueger, G., and O'Conor, G.T.: Epidemiologic and immunologic considerations on the pathogenesis of Burkitt's tumor. Recent Results in Cancer Research, Springer Verlag. Ed. E. Grundmann. (Presented at meeting in Dusseldorf, Germany by G. Krueger, Nov. 1971). In press.

B. Dr. Ralph D. Powell, Jr.:

1. Sexual maturation in olfactomized female rats. In collaboration with N. Sato, E.W. Haller,, and R.I. Henkin. Submitted for publication in Fertility and Sterility. In this study of 140 rats, removal of the olfactory bulbs at 6 days of age delayed by approximately 10 days sexual maturation as manifested by: vaginal opening, increase in ovarian weight and appearance of corpora lutea in ovaries.

2. A study of the natural history of bronchogenic carcinoma and the effect of therapy on survival in collaboration with K.B. Lamoureux, Radiation Branch, NCI. The first patients have been admitted and randomly assigned to either of two therapy protocols: radiotherapy with adjuvant therapy or radiotherapy without adjuvant therapy.

3. A study of maturation of ovarian follicles in women with decreased production of gonadotrophic hormones. In collaboration with R.L. Goldenberg, J.M. Grodin and G.T. Ross.

4. The uterine cervix as the presenting site of metastatic mammary carcinoma. Report of a case with review of the literature as well as review of relevant patient material available in the Laboratory of Pathology. In collaboration with S.H. Bennett, Surgery Department, NCI.

5. Diffuse amyloidosis of the tracheobronchial tree. In collaboration with A.J. Cook. A clinicopathologic study with emphasis upon the bronchographic findings.

6. Pulmonary aspergillosis: A clinicopathologic study correlating radiographic and postmortem anatomic findings. In collaboration with A.J. Cook and L.M. Buja.

7. I anticipate resumption of studies in the area of pulmonary pathology and physiology.

8. Other activities:

(a) I plan to continue work on the International Reference Center for Histopathological Nomenclature and Classification of Uterine and Placental Tumours, World Health Organization.

C. Dr. Hugh Bonner:

1. Case report: Coexisting lymphoid infiltration and amyloidosis of lung in Sjogren's syndrome. In collaboration with G.W. Geelhoid and T.M. Tarpley, Jr. Submitted to J. Amer. Med. Ass.

2. Chronic meningitis caused by cladosporium trichoides with collaboration with J.E. Bennett, A.E. Jennings, and R.I. Lopez. Submitted to Ann. Intern. Med.

3. Induction and effect of chemotherapy on lymphoreticular neoplasms induced in owl monkeys by Herpes virus saimiri. In collaboration with Richard Adamson and Pelayo Correa.

4. Histologic changes seen in oral pedicle flaps. In collaboration with Glenn Geelhoed and Howell Archard.

5. Oral melanoma: Case report and review of the literature. In collaboration with Randall J. Lewis.

6. Malignant lymphoma complicating polycythemia vera: Case report and review of the literature. In collaboration with Stephen H. Rosenoff.

7. Massive infarction of the spleen with thrombosis of the splenic and portal veins occurring in chronic myelogenous leukemia: Case report and review of the literature. In collaboration with Alan Rosenthal.

8. Non-lymphomatous malignancy complicating Hodgkin's disease. Report of three cases and review of 437 cases of Hodgkin's disease from the National Cancer Institute. In collaboration with James Arseneau, Robert Sponzo, Lowell E. Schnipper, George Canellos, and Vincent DeVita. (Will be submitted to New Eng. J. Med.).

9. Histiocytic medullary reticulosis: Report of a case and review of the literature. In collaboration with Stephen Newman.

D. Dr. L. Max Buja:

1. Daunomycin cardiotoxicity -- In collaboration with the Pathology Section, NHLI and Medicine Branch, NCI.

2. Ultrastructural cytochemistry of basophilic degeneration of myocardium and other stored carbohydrate-containing materials. In collaboration with Section of Pathology, NHLI.

3. Topography of early human arteriosclerosis. In collaboration with Section of Pathology, NHLI and Section of Experimental Atherosclerosis, NHLI.

4. Clinicopathologic studies in Sipple's syndrome. In collaboration with the Surgery Branch, NCI, and Experimental Therapeutics Branch, NHLI.

5. Radiographic and pathologic correlative studies in pulmonary Aspergillosis. In collaboration with the Diagnostic Radiology Department, CC.

6. Histaminase activity: A biochemical marker for medullary carcinoma of the thyroid. In collaboration with S.B. Baylin, M.A. Beavern, and H.R. Keiser. This has been submitted to Clinical Research.

7. Application of periodate-thiocarbohydrazide-silver proteinate method for the ultrastructural cytochemistry of stored materials in diseases of carbohydrate and glycolipid metabolism. In collaboration with V.J. Ferrans, NHLI. Submitted to Fourth International Congress of Histochemistry and Cytochemistry.

8. Coexistent infantile neuroaxonal dystrophy and "senile changes" in a young adult: A clinical, light and ultrastructural microscopic study. In collaboration with M.P. Valsamis and H.M. Wisniewski.

Publications:

1. Buja, L.M., Levitsky, S., Souther, S.G., Ferrans, V.J., Roberts, W.C., and Morrow, A.G.: Acute and chronic effects of normothermic anoxia on canine hearts: Light and electron microscopic evaluation. Circulation 43: I 44-50, 1971.

2. Levitsky, S., Mullin, E.M., Sloane, R.E., Ferrans, V.J., Buja, L.M., and Morrow, A.G.: Effects of a hyperosmotic perfusate on extended preservation of the heart. Circulation 43: I 124-129, 1971.

3. Ferrans, V.J., Buja, L.M., Levitsky, S., and Roberts, W.C.: Effect of hyperosmotic perfusate on ultrastructure and function of the isolated canine heart. Lab. Invest. 24: 265-272, 1971.

4. Ferrans, V.J., Buja, L.M., Roberts, W.C., and Fredrickson, D.S.: The spleen in type I hyperlipoproteinemia: histochemical biochemical, microfluorimetric, and electron microscopic observations. Amer. J. Path. 64: 67-96, 1971.

5. Buja, L.M., Freed, T.A., Berman, M.A., Morrow, A.G., and Roberts, W.C.: Pulmonary alveolar hemorrhage: a common finding in patients with severe cardiac disease. Amer. J. Cardiol. 27: 168-172, 1971.

6. Buja, L.M., and Roberts, W.C.: Iron in the myocardium: its functional and etiologic significance. Amer. J. Med. 51: 209-221, 1971.

7. Buja, L.M., Ali, N., Fletcher, R.D., and Roberts, W.C.: Stenosis of the right main pulmonary artery: a complication of acute dissecting aneurysm of the ascending aorta. Amer. Heart J. 83: 89-92, 1972.

8. Ferrans, V.J., Buja, L.M., and Roberts, W.C.: Origin and significance of glycogen-rich cytoplasmic bodies in cardiac muscle cells. Arch. Path. (In press)

9. Ferrans, V.J., Buja, L.M., Levitsky, S., Williams, W.H., and Roberts, W.C.: Cardiac preservation: a morphologic study. (Proc. of the Third International Study Group for Cardiac Metabolism held in Stowe, Vermont, June 1970). In press

10. Ferrans, V.J., Hibbs, R.G., Cipriano, P.R., and Buja, L.M.: A histochemical and electron microscopic study of norepinephrine-induced myocardial necrosis. (Proc. of the Third International Study Group for Cardiac Metabolism held in Stowe, Vermont, June 1970). In press

11. Roberts, W.C., and Buja, L.M.: The incidence and significance of coronary arterial thrombi and other observations in fatal acute myocardial infarction: a study of 107 necropsy patients. Amer. J. Med. (In press)

12. Baylin, S.B., Beaven, M.A., Buja, L.M., and Keiser, H.R.: Histaminase activity: a biochemical marker for medullary carcinoma of the thyroid. Amer. J. Med. (In press)

13. Buja, L.M., Ferrans, V.J., and Levitsky, S.: Occurrence of intramitochondrial glycogen in canine myocardium after prolonged anoxic cardiac arrest. J. Molec. Cell. Cardiol. (In press)

E. Dr. Joseph M. Davie:

1. Genetic control of the immune response of inbred guinea pigs to 2, 4-dinitroplenyl-guinea pig albumin. Frequency of antigen-binding lymphocytes and avidity of antibodies secreted by plaque-forming cells. In collaboration with W.E. Paul and I. Green. (Submitted to J. Immun.)

2. Preferential tolerization of high affinity "B" cells. In collaboration with W.E. Paul, D.H. Katz, and B. Benacearraf. Will be submitted to J. Exp. Med.

3. Hapten-specific tolerance induced in "B" cells fails to affect delayed hypersensitivity. In collaboration with B. Cohen, and W.E. Paul. Will be submitted to J. Immun.

4. Selective proliferation of precursors of high affinity antibody-forming cells during maturation of the immune response. In collaboration with W.E. Paul. Will be submitted to Nature.

5. The induction of tolerance is independent of the C4 complement system. In collaboration with B. Cohen, and I. Green. Will be submitted to J. Immun.

F. Dr. Elaine Jaffe:

1. Case report of a case of yellow nails, bronchiectases, and lymphoedema, associated with angiopathic amyloidosis in collabo ation with Dr. John Gill, NHLI.

2. Vascular invasion in Hodgkin's disease in collaboration with Kent Lamoureux, NCI, and Costan Berard.

3. Review of reticulum cell sarcoma within spectrum of non-Hodgkin's lymphoma in collaboration with Philip Schein, NCI and Costan Berard.

4. Wiscott-Aldrich syndrome, clinicopathologic review, in collaboration with Dr. Costan Berard.

5. Review of the extramedullary manifestation of chronic myelogenous leukemia and chronic myelogenous leukemia at autopsy. In collaboration with Dr. Costan Berard.

6. Histochemical, electron microscopical and light microscopical study of adrenal adenomas in collaboration with Drs. Donald Henson and L. Max Buja.

7. Calcification in a benign non-functioning adrenal adenoma. In collaboration with Michael Vermess, Diagnostic Radiology, CC, Lionel Schour, NCI. Submitted to Brit. J. Radiol.

Publications:

1. Shimkin, P., Soloway, M., and Jaffe, E.: Metastatic melanoma to the gallbladder. Amer. J. Roentgenol. (In press)

2. Bobrow, S.N., Jaffe, E., and Young, R.C.: Anuria and acute tubular necrosis associated with gentamycin and cephalothin. J. Amer. Med. Ass. (In press)

G. Dr. Ronald A. DeLellis:

1. Glenner, G.G., Page, D., Isersky, C., Harada, M., Cuatrecasas, P., Eanes, E.D., DeLellis, R.A., Bladen, H.A., and Keiser, H.R.: Murine amyloid fibril protein: isolation, purification, and characterization. J. Histochem. Cytochem. 19: 16-29, 1971.

2. Kruger, G.R., Berard, C.W., DeLellis, D.A., Graw, R.G. Jr., Yankee, R.A., Leventhal, B.G., Rogentine, G.N., Herzig, G.P., Halterman, R.H., and Henderson, E.S.: Graft-versus-host disease: Morphologic variation and differential diagnosis in 8 cases of HL-A matched bone marrow transplantation. Amer. J. Path. 63: 179-197, 1971.

3. Schall, G.L., Zeiger, L., Primack, A., and DeLellis, R.A.: Uptake of ^{85}Sr by an osteosarcoma metastatic to lung. J. Nuclear Med. 12: 131-133, 1971.

4. DeLellis, R.A., and Roth, J.A.: Norepinephrine in a glomus jugulare tumor: Histochemical demonstration. Arch. Path. 92: 73-76, 1971.

5. DeLellis, R.A.: Iron containing plasma cells in a healed recluse spider bite. Arch. Path. 92: 254, 1971.

6. Schall, G.L., Larson, S.M., and DeLellis, R.A.: Pathogenesis of the positive bone scan and its implications for the detection of metastatic osteosarcoma. Surg. Oncology 3: 673, 1971.

7. DeLellis, R.A.: Formaldehyde-induced fluorescence technique for the demonstration of biogenic amines in diagnostic histopathology. Cancer 28: 1704-1710, 1971.

8. Isersky, C., Page, D.L., Cuatrecasas, P., DeLellis, R.A., and Glenner, G.G.: Murine amyloidosis: Immunologic characterization of amyloid fibril protein. J. Immun. 107: 1690, 1971.

9. Shimkin, N., DeLellis, R.A., Carolla, R., and Weinstein, M.A.: Radiological features of graft versus host disease. Radiology (In press).

H. Dr. Joel A. Roth:

1. Roth, J.A., Siegel, S.E., Levine, A.S., and Berard, C.W.: Fatal recurrent toxoplasmosis in a patient initially infected via a leukocyte transfusion. Amer. J. Clin. Path. 56: 601-605, 1971.

Serial No. NCI 857
1. Laboratory of Pathology
2. Surgical Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Interstitial Pulmonary Emphysema: Acute and chronic morphological and physiological changes induced by elevation of intratracheal air pressure

Previous Serial Number: Same

Principal Investigator: Ralph D. Powell, Jr., M.D.

Other Investigators: Edgar J. Caldwell, M.D., and John P. Mullooly, Ph.D.

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total: 1/12
Professional: 1/24
Other: 1/24

Project Description:

Objectives:

1. To determine under standard conditions the amount of airway pressure necessary to produce dissection of air into the pulmonary interstitial space.

2. To characterize the lesion morphologically with regard to location, extent, shape, etc.

3. To study the concomitant physiologic changes and determine the pathogenetic significance of such variables as: pulmonary artery pressure, pleural space pressure, lung volume and absolute airway pressure.

4. To expose animals on a chronic basis to elevated airway pressures to determine if such stress will induce emphysema of the type commonly seen in adult humans and to study the evolution of the interstitial emphysema.

5. To compare the lesions occurring in rabbits with those occurring in man to shed light on pathogenetic mechanisms, especially those operating in man.

Major Findings:

1. The incidence of interstitial emphysema is directly related to the applied airway pressure and is high above 50 cm. of water pressure.

2. The incidence of interstitial emphysema increased directly with the degree of expansion (volume) of the lung.

3. The incidence of interstitial emphysema does not seem to be related to the pulmonary artery pressure.

4. The lesion is found less frequently in animals following chronic exposure than following acute exposure at comparable pressures.

5. The lesion in man and rabbits is morphologically very similar.

Significance:

1. In the rabbit, interstitial pulmonary emphysema with significant tissue destruction is induced by airway pressures lower than those normally generated by coughing in the human or than those often created during therapeutic intermittent positive pressure breathing.

2. The observation that the incidence of interstitial emphysema increases with the degree of expansion of the lung, supports the hypothesis that pressure in the interstitial space falls as the degree of expansion of the lung increases, and the hypothesis that the essential cause of interstitial emphysema is the creation of a large pressure gradient between alveoli and the interstitial space.

3. During chronic exposure, the lesion in rabbits appears to heal and the animal becomes more resistant to induction of the lesion.

4. The definite morphological and probably physiological similarities between the lesion in rabbits and in man suggest that preventive measures such as limiting airway pressure elevation and degree of lung expansion may be effective methods of reducing the incidence of the lesion in man.

Proposed Course:

1. Begin in vitro experiments to define more precisely the relationship of lung volume and pulmonary artery pressure to incidence of interstitial emphysema.

2. Develop techniques for measuring pressure in the interstitial space to permit monitoring of this pressure during changes in lung volume, etc.

3. Determine how much and what types of functional disturbances of respiration are produced by interstitial emphysema.

4. Establish more precisely the morphological and physiological similarities between the lesion in man and rabbits.

Serial No. NCI 859
1. Laboratory of Pathology
2. Surgical Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Renal biopsy in lupus nephritis: A statistical analysis of variability of disease at different sites within single kidneys

Previous Serial Number: Same

Principal Investigator: Ralph D. Powell, Jr., M.D.

Other Investigators: Roger L. Black, M.D., Watson Buchanan, M.D., William M. O'Brien, M.D., and Hugh M. Pettigrew

Cooperating Units: None

Man Years (July 1, 1971 through June 30, 1972)

Total:
Professional:
Other:

Project Description:

Objectives:

Work on this project has been almost completely suspended during the current year but is expected to progress more rapidly during the coming year.

Serial No. NCI 555
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Ultrastructural and Autoradiographic Studies of Membrane Structures Associated with Arbovirus Replication

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: R.M. Friedman, M.D.; I.K. Berezesky, B.S.; J.G. Levin, Ph.D.; and N. Young, M.D.

Cooperating Units: Channing Laboratory, Boston City Hospital

Man Years (July 1, 1971 - June 30, 1972)

Total: 1
Professional: 1
Other:

Project Description:

Objectives: To examine the role of membranes in arbovirus replication.

Major Findings: In tissue cultures and experimental animals ultrastructurally unique cytoplasmic membranes have been identified in cells infected by group A arboviruses. These membranes, designated type 1 cytopathic vacuoles (CPV-1), are sites of virus RNA replication. This was demonstrated by high resolution autoradiography and cell fractionation.

As infection progresses, arbovirus nucleoids accumulate in the cytoplasm and mature by budding through other host cell membranes, either cytoplasmic vacuoles or endoplasmic cisternae, or at the cell surface. Our studies have demonstrated both pathways of virus envelopment in vivo and in vitro.

Intracytoplasmic membranous structures of a unique type were associated with the replication of three group A arboviruses. The structures, referred to as type 1 cytopathic vacuoles (CPV-1), were membrane-limited and characteristically lined by regular membranous spherules measuring 50 nm in diameter. Detection of CPV-1 coincided with the time of rapid virus growth in several different cell types and preceded the accumulation of virus nucleocapsids. Counts of CPV-1 remained in a similar range even when the multiplicity of infection was varied over 100-fold. Inhibition of cellular RNA and protein synthesis by actinomycin D did not decrease the counts of

CPV-1; however, biogenesis of CPV-1 was limited when viral replication was inhibited by inhibitors of viral RNA synthesis (guanidine) or of viral protein synthesis (cycloheximide). It was concluded that formation of CPV-1 resulted from a virus-specified modification of pre-existing host cell structures. Cytochemistry suggested that Golgi cytomembranes might be involved.

When cytoplasmic extracts of cells infected with an arbovirus were subjected to isopycnic centrifugation in discontinuous sucrose gradients, seven distinct bands were usually formed. The four upper bands contained predominantly smooth membranes and the lowest band was enriched in rough endoplasmic reticulum. One fraction (fraction 5), banding at a density of 1.16 gm/cm^3, was found to be heavily enriched in pulse-labeled ribonucleic acid (RNA), viral RNA polymerase, and viral RNA forms associated with RNA replication. This fraction evidently contained a membrane-associated viral replication complex. Fraction 5 was also consistently enriched with unique membranous structures previously observed in intact cells as type 1 cytopathic vacuoles (CPV-1). When the CPV-1 in fraction 5 were isolated from cells briefly incubated with ^{3}H-uridine and ^{3}H-adenosine prior to cell disruption, a large proportion were found to be labeled by high resolution autoradiography. Thus, ultrastructural, biochemical and biological evidence were all consistent with the interpretation that CPV-1 membranes represent a significant element of the arboviral replication complex.

Significance: Identification and study of membranous structures associated with arbovirus replication contributes basic information to virology and molecular biology. The group A arboviruses include several important pathogens and studies in progress are providing basic information concerning the pathogenesis of infections.

Proposed Course: The significance and fate of virus replicative membranes will be examined further utilizing cell fractionation techniques, high resolution autoradiography, cytochemistry and conjugated antibodies. The course of virus development will be manipulated with specific inhibitors.

Serial No. NCI 555a
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Ultrastructural and Biological Studies of Effects of Rifamycin Derivatives on Poxvirus Infections

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: B. Moss, M.D.; E. Rosenblum, B.S.; and S.J. Mims, B.S.

Cooperating Units: Laboratory of Biology Viruses, NIAID

Man Years (July 1, 1971 - June 30, 1972)

Total: 1 1/2
Professional: 1 1/2
Other:

Project Description:

Objectives: 1. To examine the effects of rifamycin derivatives on molecular events in poxvirus assembly. 2. To examine the structure and formation of poxvirus membranes. 3. To study events in poxvirus morphogenesis.

Major Findings: The antibiotic rifampicin blocks poxvirus assembly. Morphogenesis is interrupted at a stage prior to formation of virus envelopes. Viral membrane which accumulate in the presence of rifampicin lack a normal outer coating. When the drug is removed, the morphologic effect is rapidly reversed. The specific effect of rifampicin on the formation of the vaccinia viral envelope was reversed within 10 minutes even in the presence of NaF or inhibitors of protein or nucleic acid synthesis. Further maturation of some virus particles, which were characterized by their ultrastructure and isolated by sucrose gradient sedimentation, proceeded in the presence of inhibitors of protein synthesis. In contrast, addition of NaF or acinomycin D almost completely blocked the development of mature virus particles. Rifampicin acted specifically at the stage of envelope formation and did not prevent later steps in maturation.

A rifampicin-resistant vaccinia virus mutant was isolated from wild-type virus stocks. Growth of the mutant was inhibited only slightly at concentrations of rifampicin which completely prevented the growth of drug-sensitive virus, and at no concentration tested was there a detectable effect on viral envelope formation. The mutant virus was capable of rescuing wild-type during

mixed infection in the presence of rifampicin; after simultaneous infection the ratio of genomes in the progeny reflected that of the original inoculum. Superinfection experiments demonstrated that heat-inactivated mutant was capable of efficiently rescuing wild-type virus and that extensive replication of the mutant genome was not required for this. Characteristic rifampicin blocked viral forms, normal maturing forms, and intermediate structures were seen in the same ultrathin cell sections after simultaneous infection with low multiplicities of mutant and wild-type virus. In cells simultaneously infected with high multiplicities of mutant and wild-type virus, rifampicin had no detectable effect on viral envelope formation.

Membrane-limited structures, resembling virus envelope precursors previously shown to form during the interruption of poxvirus assembly by rifampin, were also observed by electron microscopy in vaccinia-infected HeLa cells treated with a series of rifamycin derivatives. The active compounds N-demethyl rifampin, AF/DMI, and 3-formyl rifamycin SV lacked, respectively, a methyl group, the piperazine ring, and the hydrazone portion of rifampin. A vaccinia mutant selected only for resistance to rifampin was also resistant to the effect on morphogenesis produced by all of the rifamycin derivatives. We concluded that this antiviral effect was specific and was a property associated with the macrocyclic ring rather than the hydrazone containing side chain of rifampin. In addition to their effects on vaccinia morphogenesis, 3-formyl rifamycin SV and AF/DMI had unusual cytotoxic effects, including swelling of mitochondria.

Significance: The action of rifamycin antibiotics on poxvirus assembly is unique and provides a novel approach to studies of the macromolecular organization of viruses and of virus membrane specialization. Analysis of poxvirus membrane development may lead to further understanding of membrane bioassembly processes. Studies of the rifamycin antibiotics are of broad fundamental importance in developing therapeutically useful antiviral agents.

Proposed Course: Events in the bioassembly of poxvirus envelopes will be examined utilizing rifampicin as a probe. The molecular pharmacology of the specific antiviral action will be explored using a selected series of rifamycin derivatives.

Publications:

1. Moss, B., Rosenblum, E.N., and Grimley, P.M.: Assembly of vaccinia virus particles from polypeptides made in the presence of rifampicin. Virology 45: 123-134, 1971.

2. Moss, B., Rosenblum, E.N., and Grimley, P.M.: Assembly of virus particles during mixed infection with wild-type vaccinia and a rifampicin-resistant mutant. Virology 45: 135-148, 1971.

3. Grimley, P.M., and Moss, B.: Similar effect of rifampin and other rifamycin derivatives on vaccinia virus morphogenesis. J. Virol. 8: 225-231, 1971.

4. Grimley, P.M.: High voltage electron microscopy of poxvirus nucleoid material. In Arceneaux, C.J. (Ed.): Proceedings of Electron Microscopy Society of America. Baton Rouge, La., Claitor's Publishing Div., 1971, pp. 380-381.

Serial No. NCI 577
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Ultrastructural and Biologic Studies of Tubuloreticular Structures in Neoplastic and Virus-infected Cells

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: J. Decker, M.D., Z. Schaff, M.D.; D. Henson, M.D.; H. Michelitch, B.S.; and M. Frantz, B.S.

Cooperating Units: Arthritis and Rheumatism Branch, NIAMD

Man Years (July 1, 1971 - June 30, 1972)

Total: 1
Professional: 1
Other:

Project Description:

Objectives: To evaluate the significance of tubuloreticular structures in neoplastic cells, virus-infected cells, and cells of patients with connective tissue diseases.

Major Findings: Tubuloreticular structures were found in the circulating lymphoid cells of patients with systemic lupus erythematosus and in some related diseases of connective tissue. They resembled structures previously described in glomerular endothelium of these patients. Tubules measured 220-240 Angstroms and were located in the granular endoplasmic reticulum.

Significance: The tubuloreticular structures are often observed in the cells of patients with systemic lupus erythematosus or Sjögren's disease as well as in lymphoid cell cultures derived from normal, leukemic, cystinotic and lymphoma patients. Thus, they appear to have a broad biologic significance, and may be related to virus infections or transformation.

Proposed Course: The nature of tubuloreticular structures in connective tissue disease and in neoplastic cells will be investigated by a variety of techniques including cytochemistry and immuno-electron microscopy.

Serial No. NCI 582
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Pathogenesis of Chronic Mouse Cytomegalovirus Infection in Lymphoid Organs

Previous Serial Number: None

Principal Investigators: Donald E. Henson, M.D.; Alfonzo J. Strano, M.D.; and Margery Slotnik

Other Investigators: None

Cooperating Units: Armed Forces Institute of Pathology

Man Years (July 1, 1971 - June 30, 1972)

Total: 1
Professional:
Other:

Project Description:

Objectives: Study the mechanisms of latent murine cytomegalovirus infection in the lymphoid tissue of mice chronically infected with this virus.

Methods Employed: Tissue culture, immunofluorescence, electron microscopy.

Significance: The mouse cytomegalovirus causes chronic infection in the submaxillary glands, lymph nodes, and spleens of many strains of mice. Infection in the lymph nodes and spleen probably occurs in lymphocytes. The murine cytomegalovirus belongs to the herpesvirus group of animal viruses, along with the Epstein-Barr virus and Herpesvirus saimiri. Consequently, a study of murine cytomegalovirus infections in lymphocytes may help solve some of the problems regarding other herpesvirus infections of lymphocytes and relationship of chronic infection to lymphomas.

Proposed Course: Investigate latent cytomegalovirus infection in lymphoid tissue.

Publications:

1. Henson, D., and Strano, A.J.: Mouse cytomegalovirus: Necrosis of infected and morphologically normal submaxillary gland acinar cells during termination of chronic infection. Amer. J. Path. (in press).

2. Henson, D., Strano, A.J., Slotnik, M., and Goodheart, C.: Mouse cytomegalovirus: Isolation from lymphoid tissue of chronically infected mice. Proc. Soc. Exp. Biol. Med. (in press).

Serial No. NCI 583
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunological, Biological, and Ultrastructural Studies of Leucocytes Infected with Herpes-type Viruses

Previous Serial Number: 863c

Principal Investigators: Donald E. Henson, M.D.; Philip M. Grimley, M.D.; and Margery Slotnik

Other Investigators: A. Sullivan, M.D.

Cooperating Units: Arthritis and Rheumatism Branch, NIAMD

Man Years (July 1, 1971 - June 30, 1972)

Total: 1
Professional:
Other:

Project Description:

Objectives: 1) Develop immunologic and ultrastructural techniques for examination of surface antigens and receptor sites on human leucocytes.

2) Study effects of overt and covert infections of herpes-type viruses on distribution and types of surface receptors on leucocytes.

Major Findings: 1) Hybrid antibody technique was developed and utilized to label immunoglobulin E surface receptors on human basophils.

2) Direct antibody labeling technique has been developed to localize herpesvirus antigen in cultured lymphocytes.

Significance: Much evidence indicates that the lymphoid system is a target organ for herpes-type viruses. Development of immunologic and ultrastructural techniques will aid in our understanding of virus expression in leucocytes in clinical conditions such as lymphomas or lupus erythematosis.

Proposed Course: Continue development of methods and application as stated in objectives.

Publications:

1. Sullivan, A.L., Grimley, P.M., and Metzger, H.: Electron microscopic localization of immunoglobulin E on the surface membrane of human basophils. J. Exp. Med. 134: 1403-1416, 1971.

Serial No. NCI 585
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Histological Evolution of In Situ Carcinoma in the Vagina

Previous Serial Number: None

Principal Investigators: Donald E. Henson, M.D.; and Peyton T. Taylor, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/4
Professional: 1/4
Other:

Project Description:

Objectives: Clinical-pathological study of women with multicentric squamous cell cancers in the ectogenital epithelium.

Major Findings: Histological studies made on women with sequential vaginal biopsies have demonstrated progression from severe epithelial atypia, through dysplasia, to carcinoma in situ. As carcinoma of the cervix, in situ vaginal tumors have an invasive potential. Lesions seem to enlarge by intra-epithelial invasion.

Significance: Many women with cancer of the uterine cervix subsequently develop epithelial cancers in the vagina and/or vulva. These multiple cancers have been considered to arise in an epithelial region predisposed to malignancy. This region has a common cloacal origin embryologically.

Proposed Course: Project will continue.

Serial No. NCI 861
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Morphological, Biochemical and Biological Studies of Hormonally Active Neoplasms

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: N.A. Wivel, M.D. (NCI-4833)

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/4
Professional: 1/4
Other:

Project Description:

Objectives: To examine effects of viral transformation on neoplastic endocrine cells in vitro.

Major Findings: Two tumors, medullary carcinomas from human thyroid glands, were used to establish tissue culture lines. These lines were assayed for alkaline phosphatase activity and were found to be consistently positive. Following viral transformation of the cell lines with SV_{40}, the alkaline phosphatase levels were markedly depressed or undetectable. When hydrocortisone was added to the in vitro system, the enzyme activity returned to preinfection levels. This increase in alkaline phosphatase was dependent on the continuous presence of steroid; when steroid was withdrawn there was a loss of activity to pretreatment levels. No increase in enzyme levels was detected in uninfected tumor cells treated with hydrocortisone.

Significance: Findings were consistent with the hypothesis that SV_{40} may repress a cellular gene during in vitro viral transformation.

Proposed Course: To continue tissue cultures of endocrine carcinoma cells and to examine specific enzyme activities. To examine the effect of SV_{40} viral transformation on enzyme activities in the other cell systems.

Publications:

1. Wivel, N.A., and Grimley, P.M.: Reversible suppression of alkaline phosphatase in human thyroid medullary carcinoma cells transformed by SV_{40}. Proc. Soc. Exp. Biol. Med. 139: 627-630, 1972.

Serial No. NCI 863a
1. Laboratory of Pathology
2. Ultrastructural Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Ultrastructural Studies of Human Cytology Specimens

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: E.W. Chu, M.D.; I.K. Berezesky, B.S.; H.J. Michelitch, B.S.; and T.A. Wood, Jr., B.S.

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total: 1/4
Professional: 1/4
Other:

Project Description:

Objectives: To compare the ultrastructure of normal and neoplastic cells in samples of body fluids.

Major Findings: Preliminary correlation of electron micrographs with cytology specimens suggests that histiocytes and mesothelial cells in pleural fluids may be distinguished from neoplastic cells by ultrastructural features.

Significance: Results contribute basic information regarding tumor cell structure and may assist in cytodiagnosis.

Proposed Course: To continue examination of cell preparations by transmission and scanning electron microscopy.

Serial No. NCI 524
1. Laboratory of Pathology
2. Viral Oncology and Molecular Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Interferon Production and Action

Previous Serial Number: Same

Principal Investigator: Robert M. Friedman, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total:
Professional:
Other:

Project Description:

During this year Dr. Friedman has been working in the Biochemistry Department of the National Institute for Medical Research at Mill Hill, London, England, with special emphasis on a cell free protein synthesizing system. He will make a full report of his findings after he returns to the National Cancer Institute.

Serial No. NCI 531
1. Laboratory of Pathology
2. Viral Oncology and Molecular Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: In Vitro Studies of Human Malignant Tumors

Previous Serial Number: Same

Principal Investigator: Alan S. Rabson, M.D.

Other Investigators: G.T. O'Conor, M.D., E.W. Chu, M.D., Daniel Albert, M.D. (Yale University), and Saul Rosen, M.D. (NIAMD)

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total: 1
Professional: 2/3
Other: 1/3

Project Description:

Objectives: To study biological properties of human lymphoma, sarcoma, carcinoma and retinoblastoma cells in vitro and to attempt to isolate oncogenic viruses from these cells.

Methods Employed: Standard tissue culture, virologic and pathologic techniques are used.

Major Findings: We have continued to isolate cell lines from human tumors and to attempt to identify and isolate oncogenic viruses from these cells. Thus far no human oncogenic viruses have been isolated.

We have established a cell line from a human bronchogenic carcinoma. The patient had signs and laboratory evidence of increased gonadotropins and the tumor in culture has been producing human chorionic gonadotropin in the culture fluids for 10 months. This work is being done in collaboration with Dr. Saul Rosen, NIAMD.

We have isolated an epithelial cell line from a human carcinoma of the cervix which has been extensively studied for presence of Herpesvirus hominis and its antigens but no virus or viral antigens have been detected thus far.

In collaboration with Dr. Albert (Yale University) we have continued our studies of human retinoblastoma. Several new cell lines have been established but no viral particles have been seen by electron microscopy and no viruses have been isolated in culture.

In collaboration with Dr. R.L. Kirschstein (DBS) we have been attempting to isolate viruses from mammary gland tissue of monkeys during treatment with oral contraceptives. One of 6 monkeys treated has developed an infiltrating duct carcinoma of the breast. Attempts to establish this tumor in culture were unsuccessful and no evidence of virus was found.

Significance: The importance of the search for possible viral etiology of human malignant tumors is evident. In addition, any information about the biological properties of human tumor cells may be of importance in our understanding of the lymphoma problem.

Proposed Course: Attempts to isolate viruses from human malignant tumors using a variety of techniques which have been successful in the isolation of oncogenic viruses in animal systems will be continued. Studies of factors involved in human chorionic gonadotropin production in vitro in lung cancer cells will be initiated.

Publications:

1. Albert, D.M., Tso, M.O.M., and Rabson, A.S.: In vitro growth of pure cultures of retinal pigment epithelium. Arch. Ophthal. (in press).

2. Albert, D.M., Tso, M.O.M., and Rabson, A.S.: Experimental malignant tumors from retinal pigment epithelium. Arch. Ophthal. (in press).

3. Kirschstein, R.L., Rabson, A.S., and Rusten, G.W.: Infiltrating duct carcinoma of the mammary gland of a rhesus monkey after administration of an oral contraceptive: A preliminary report. J. Nat. Cancer Inst. 48: 551-556, 1972.

Serial No. NCI 532
1. Laboratory of Pathology
2. Viral Oncology and Molecular Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Oncogenic DNA Viruses

Previous Serial Number: Same

Principal Investigator: Alan S. Rabson, M.D.

Other Investigators: E. Chu, M.D.; R.L. Kirschstein, M.D.; G.T. O'Conor, M.D., P. Periman, M.D.; and R. Chambers, M.D. (Georgetown University)

Cooperating Units: None

Man Years (July 1, 1971 - June 30, 1972)

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

Objectives: To study some biological properties of DNA viruses with proven or possible oncogenic activity.

Methods Employed: Standard virologic and pathologic methods have been used.

Major Findings: Studies of herpesviruses with possible oncogenic properties have continued with emphasis on Herpesvirus saimiri and human cytomegaloviruses. Studies of the MLC-1 cell line of marmoset lymphoid cells derived from a marmoset with a lymphoma-like lesion induced by H. saimiri have continued. Approximately 0.5% of the cells in the culture now can be induced to release H. saimiri on contact with monolayer cultures of susceptible "indicator cells" such as the Vero line of African green monkey kidney. Attempts are being made to increase the number of "inducible cells" by treatments with radiation, hormones, argenine deficiency, temperature changes, metabolic inhibitors and drugs such as cytochalasin B. Thus far a number of these treatments result in small but inconsistent increases and the mechanisms of induction and repression are being explored.

In collaboration with Dr. Chu, cytogenetic studies of the marmoset lymphoid cell have been carried out. Since marmosets are hematopoietic chimeras, those that are the product of a twin pregnancy with a fraternal twin of the opposite sex will contain both male and female hematopoietic

cells. The cell line was found to contain both male and female cells in equal proportions thus eliminating the possibility that the line was of monoclonal origin.

In collaboration with Dr. Phillip Periman, we have begun studies of cell fusion with the marmoset lymphoid line in an attempt to learn more about the cell-virus relationship. Marmoset-mouse hybrid cell lines have been derived from fusion of MLC-1 marmoset lymphoid cells with the RAG line of 8-azaguanine-resistant murine adenocarcinoma cells by use of Sendai virus and growth in aminopterin-containing medium. Cytogenetic studies of the hybrid lines show that the cells contain both mouse and marmoset chromosomes. Whereas the MLC-1 cells release small amounts of virus into the supernatant and contain between 0.1 to 1% of cells that can release H. saimiri on contact with Vero cells, the hybrid cells release no virus into the supernatants and between 0.01 and 0.09% of the cells release virus on contact with Vero monolayers. The absence of virus in the supernatants and decrease in number of cells plating as infectious centers in the hybrid lines suggests either that the mouse components exert some inhibitory effect on the expression of the H. saimiri genome, or that replication depends on MLC genes which have been lost. The number of cells yielding virus decreased as the hybrid lines were grown in medium free of aminopterin with loss of marmoset chromosomes suggesting that the virus in the MLC-1 cells is in some way closely associated with the chromosomes.

Methods for growth of large amounts of human cytomegaloviruses have been developed (in collaboration with Dr. Robert Chambers, Georgetown University, and Dr. James Rose of NIAID). Viral DNA has been prepared from cytomegaloviruses for use in hybridization experiments. A strain of human cytomegalovirus has been isolated from the spleen of a Clinical Center patient with a lymphoproliferative disease and the effects of this virus on human lymphoreticular cells is being studied.

Significance: The mechanisms of repression and possible integration of herpesviruses in mammalian cells and mechanisms of neoplastic transformation may be of extreme importance in understanding the role of herpesviruses in human cancer. Studies with a model system involving a primate oncogenic herpesvirus such as H. saimiri and studies of herpesviruses in human lymphoma cells may be of value in this regard.

Proposed Course: We intend to continue studies of the virus-cell relationship in the marmoset lymphoid cell line and to study factors involved in activation of the viral genome. We also intend to continue studies of the mechanisms of repression of herpesviruses in both lymphoid and non-lymphoid cell systems.

Publications:

1. Periman, P., Tyrrell, S.A., and Rabson, A.S.: Herpesvirus saimiri in marmoset-mouse hybrid cell lines. J. Nat. Cancer Inst. (in press).

2. Rabson, A.S., Tyrrell, S.A., and Legallais, F.Y.: An inhibitor of Herpesvirus hominis in extracts of cultures of Burkitt's lymphoma cells. Proc. Soc. Exp. Biol. Med. 137: 264-267, 1971.

3. Berezesky, I.K., Grimley, P.M., Tyrrell, S.A., and Rabson, A.S.: Ultrastructure of a rat cytomegalovirus. Exp. Molec. Path. 14: 337-349, 1971.

4. Albert, D.M., and Rabson, A.S.: Inhibition of ocular Herpes Simplex infection in rabbits by extracts of Burkitt's lymphoma cell cultures. Proc. Soc. Exp. Biol. Med. 138: 108-111, 1971.

5. Chambers, R.W., Rose, J.A., Rabson, A.S., Bond, H.E., and Hall, W.T.: The propagation and purification of high titer human cytomegalovirus. Appl. Microbiol. 22: 914-918, 1971.

6. Chu, E.W., and Rabson, A.S.: Chimerism in lymphoid cell culture line derived from lymph node of marmoset infected with Herpesvirus saimiri. J. Nat. Cancer Inst. 48: 771-775, 1972.

Serial No. NCI 580
1. Laboratory of Pathology
2. Viral Oncology and Molecular Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Virus-host Relationship in Arbovirus Infection

Previous Serial Number: None

Principal Investigator: Judith G. Levin, Ph.D.

Other Investigators: P.M. Grimley, M.D.; and P. Periman, M.D.

Cooperating Units: George Washington University Medical School

Man Years (July 1, 1971 - June 30, 1972)

Total: 2 1/2
Professional: 2
Other: 1/2

Project Description:

Objectives: 1. To study host cell involvement in group A arbovirus infection. 2. To identify host cell factors which may be required for viral RNA and protein synthesis.

Methods Employed: The methods employed include growth of cells in tissue culture; growth of virus in various cell lines and determination of virus titre by plaque assay; preparation of cell-free extracts for assay of viral RNA polymerase activity; polyacrylamide gel electrophoresis of viral proteins and RNA species extracted from infected cells; electron microscopy; isolation and characterization of cell hybrids.

Major Findings: 1. As a first approach to the study of the virus-host relationship, an examination of cell systems in which virus is produced with varying efficiency has been undertaken. Two L cell lines have been obtained, one which yields high titres of Semliki Forest virus (SFV) and another which produces lower yields of virus per cell. The kinetics of viral growth are quite different in the two lines and this difference is reflected in the corresponding rates of viral RNA synthesis. Other parameters such as relative proportion of viral RNA forms present during infection, levels of viral RNA polymerase etc. are also being examined in order to compare SFV-infected L cells with each other and with chick embryo fibroblast cells, heretofore the standard tissue culture system used for growth of SFV and other group A arboviruses. Similar studies are being conducted with HeLa cells which produce low amounts of SFV.

2. Ultrastructural characterization of SFV-infected L cells and HeLa cells has revealed the presence of the membranous structures characteristically found in cells infected with group A arboviruses. CPV-1 structures, which are thought to be associated with viral RNA synthesis, are particularly well preserved in L cells and appear to occur in greater numbers in cells which produce lower virus yields.

Significance: Recent findings with bacteriophage systems have provided evidence that viral enzymes contain host polypeptide subunits. It is quite likely that a similar situation exists in the animal virus systems. Clarification of host cell involvement in virus infection will lead to a greater understanding of arbovirus infection with which the work is presently concerned and is also relevant to such pressing questions as why leukemia virus is produced in some cells but not in others or why some cells are transformed by oncogenic viruses whereas others are resistant.

Proposed Course: 1. In progress: Detailed examination of the kinetics of synthesis of specific viral RNA forms in the various cell lines. Analysis of viral proteins synthesized during infection using polyacrylamide gel electrophoresis.

2. Study of biochemical events early in infection when an influence of the host cell might be expected to be most pronounced.

3. Cell hybridization experiments. Hybrids are being constructed between an L cell mutant which has been found to be a poor SFV producer and the more permissive L cell line. The hybrid cells will be subjected to the type of analysis described above and such questions as, for example, whether ability to support virus growth is a dominant trait will be explored.

4. Extension of this approach to other viral systems.

Honors: 1. Election to New York Academy of Sciences.

2. Election to American Society of Biological Chemists.

Serial No. NCI 581
1. Laboratory of Pathology
2. Viral Oncology and Molecular Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Isolation and Characterization of an Arbovirus-induced RNA Polymerase

Previous Serial Number: None

Principal Investigator: Judith G. Levin, Ph.D.

Other Investigators: T. Sreevalsan, Ph.D.; and R.M. Friedman, M.D.

Cooperating Units: Georgetown University Medical School

Man Years (July 1, 1971 - June 30, 1972)

Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

Objectives: To isolate and characterize the viral RNA polymerase synthesized in cells infected with Semliki Forest virus (SFV), a group A arbovirus.

Methods Employed: The methods employed include preparation and partial purification of extracts of infected cells, assay of enzymatic activity by measuring the incorporation of radioactivity from a ^{3}H-labeled nucleoside triphosphate (generally ^{3}H-GTP alone or in combination with ^{3}H-UTP) into acid-insoluble material, extraction of viral RNA formed *in vitro* and in infected cells, and analysis of these RNA species by polyacrylamide gel electrophoresis.

Major Findings: The Semliki Forest virus (SFV) RNA polymerase is induced during infection and occurs in infected cells as part of a membrane-bound replication complex sedimenting with the mitochondrial fraction at 13,000 x g. The properties of the enzyme e.g. resistance to high doses of actinomycin D, Mg^{++} optimum, kinetics and temperature-dependence of the reaction etc. were examined and it was found somewhat unexpectedly, that the reaction proceeds more efficiently at 28°C than at 37°C. As shown by analysis on polyacrylamide gels, the products synthesized by membrane-associated or detergent-treated enzyme are primarily the double-stranded replicative forms. Although formation of other viral RNA species (e.g. single-stranded RNA's) has not yet been detected *in vitro*, it was possible to demonstrate that all of the SFV RNA species identified in whole cells are associated with the subcellular fraction exhibiting maximum enzyme activity.

Significance: 1. The details of arbovirus RNA replication are still not completely understood. By utilizing an *in vitro* system, reactions involving RNA synthesis can be easily separated from other events in virus replication, and a more direct approach to elucidating the mechanism of the reaction(s) can be undertaken. The reactions are undoubtedly quite complex since there are at least eight species of viral RNA in infected cells and only one species of RNA in the mature virion. A clearer understanding of these reactions should add to our general knowledge of RNA metabolism in animal cells.

2. As a corollary to studies on the mechanism of enzyme action, inhibitors of the enzyme may be found. Such compounds would be potential antiviral agents with possible applications in medicine.

3. The association of the viral RNA polymerase with membranous structures in infected cells makes the study of the enzyme of more general interest since many cellular reactions (including synthesis of some proteins) normally occur on cellular membranes. In addition, other viruses such as the RNA tumor viruses also replicate on cellular membranes and the arbovirus system may be a useful model for research on these agents.

Proposed Course: 1. Parallel studies on the viral RNA polymerase induced by Sindbis virus, another group A arbovirus are now in progress.

2. More purified preparations of the enzyme and more stringent conditions for isolation of the RNA products are being employed in an effort to detect single-stranded, ribonuclease-sensitive, viral RNA species. Preliminary data indicate that this is a promising approach.

3. Detailed studies on the mechanism of enzyme action would attempt to answer such questions as: Does one enzyme catalyze formation of single-stranded and double-stranded viral RNA or are several enzymes involved? Is the synthesis of RNA strands assymetric?

4. Experiments designed to probe the nature of the association of the viral polymerase and the membranous structures to which it is attached.

Publications:

1. Levin, J.G., and Friedman, R.M.: Analysis of arbovirus ribonucleic acid forms by polyacrylamide gel electrophoresis. J. Virol. 7: 504-514, 1971.

SUMMARY REPORT

RADIATION BRANCH

NATIONAL CANCER INSTITUTE

July 1, 1971 through June 30, 1972

The highlight of our research program during the past year has been continued progress in the field of integrated therapy and these accomplishments have major significance with respect to the overall goal of cancer control. Traditionally, therapeutic approaches to the various neoplastic diseases have emphasized a single treatment modality. Unfortunately, universal experience has repeatedly demonstrated that local surgery or radiation therapy may serve to eradicate the primary tumor but the patient eventually succumbs to metastatic disease. Conversely, systemic treatment by means of chemical agents or immunologic manipulation has often been unrewarding because of the presence of a prohibitively large tumor cell burden. That single modality treatment too frequently has serious inherent weaknesses has strengthened our conviction that the strategy of the future, aside from the ultimate goal of prevention, must be the application of combined treatment approaches whenever indicated. The Radiation Branch is unique among the clinical units of the National Cancer Institute in having directed its primary effort toward the coordinated exploitation of multiple therapeutic tools and the term "multi-system therapy" or MST has been adopted in reference to this approach.

Several ingredients are requisite for the effective application of the MST approach in cancer therapeutics. These include a sound fundamental understanding of the natural history of each tumor, clinical experience which provides an accurate estimate of the "success rate" with a single modality therapy for the various clinical presentations within the spectrum of this natural history, and the availability of efficient methods for discerning the location of any given patient's disease process within the natural history for that cancer. These ingredients are fortunately available in certain instances as illustrated by the example of Hodgkin's disease.

Investigations on Hodgkin's disease by the Radiation Branch have markedly contributed to the understanding of the natural history of the disease and its therapeutic management. Our program has yielded excellent, and to date unparalleled, long-term survival rates for patients with clinically localized or regionally advanced involvement at the time of diagnosis. And by prospective study, it has become possible to identify those selected patients with apparently "early" disease who are in fact at high risk of failing to be cured with radiation therapy. This has been accomplished by correlating clinical and histopathologic determinants with biologic behavior as measured by disease-free survival. These high risk patients thereby constitute an ideal population for MST and a program of combined radiation therapy and chemotherapy is now producing superior results compared to radiation therapy alone. However, just as single modality treatment is not optimal for certain patients, it should be recognized that MST is not required for every patient with Hodgkin's disease.

With many other forms of cancer, diagnostic techniques are lacking for the reliable assessment of the biologic extent of disease, the natural history of the neoplasm is poorly defined, or the diagnosis is commonly established at that point in the natural history of disease when metastatic dissemination has already taken place. Under these circumstances, therapy must perforce be empiric and employ a MST approach to maximize the chances for therapeutic success. We have investigated this contingency in experimental therapeutics with the adoption of Ewing's sarcoma as a model system. Ewing's sarcoma has a striking propensity for early dissemination and is rarely cured by aggressive treatment confined to the primary tumor site. Our MST approach, involving various permutations of surgery, radiation therapy, and chemotherapy, has succeeded in dramatically improving the usual dire prognosis by quadrupling the disease-free survival rate.

This combined treatment program for Ewing's sarcoma has conceptual implications for those types of cancer with the highest national incidence and mortality rates. Thus, as a natural outgrowth of the Ewing's study, we have initiated pilot research studies on cancer of the lung based on this philosophy of integrated therapeutics. Furthermore, it seems mandatory to develop an "in depth" attack on lung cancer and to this end, an "ad hoc" task force is being assembled to create a comprehensive program for the study of lung cancer. In the near future, a detailed proposal will be forthcoming, accompanied by a request for administrative committment of resources including the specific assignment of Clinical Center beds for intramural aspects of this program. The desirability of centralizing a lung cancer program within the Radiation Branch is obviously supported by documented evidence that dedication exists to the integrated study and coordinated study of cancer. Such has been demonstrated not by "lip service" but rather solidly demonstrated by the careful design, meticulous prosecution, and successful completion of relevant clinical programs in recent years.

Whereas other areas of clinical research and investigation are described in detail under the Individual Project Report section of the annual report, the remainder of our program will not be reviewed here. Comment is deserving, however, that the Radiation Branch continued to provide major support to other areas of the Institute as witnessed by approximately 5,000 non-clinical radiation exposures performed during the past year and in excess of 200 patients having been seen in consultation.

Finally, notation is compelled with respect to the facilities of the Radiation Branch. There has been a continuous and relatively constant escalation in the level of clinical activity during the past six years. The number of individual patient visits during the past year, consisting primarily of outpatient treatments, totaled 6500 as contrasted to 2800 in 1965. The available facilities are becoming stressed by this clinical volume and proposed efforts such as the study on cancer of the breast will have a further impact leading to logistical decompensation. There is an imperative need for an expanded clinical department within the next several years, based primarily on the increasing demand for outpatient services.

Serial No. NCI-650 (c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Service Radiation Therapy

Previous Serial Number: Same

Principal Investigator: Thomas C. Pomeroy, M.D.

Other Investigators: Frederick L. Faw, M.S.; Dwight W. Glenn, M.S.; Marilyn K. Glover, R.N., and Sandra K. Johnson, R.N.

Cooperating Units: None

Man Years:

Total:	3/2
Professional:	2/2
Other:	1/2

Project Description:

Objectives: To provide expert radiotherapy consultation and treatment for Clinical Center patients admitted to services other than the Radiation Branch of the National Cancer Institute.

Patient Material: During the twelve month period preceding this report, 204 patients were formally seen in consultation. Advice and management of additional patients was provided on an emergency or "ad hoc" basis.

Methods Employed: The majority of patients were irradiated with Cobalt teletherapy following appropriate evaluation and treatment planning as indicated.

Major Findings: Aside from results on research patients as described in project reports from other institutes, the response to radiation therapy has been consistent with that reported from other major centers.

Proposed Course: To continue.

Honors and Awards: None

Publications: None

Serial No. NCI-958(c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Study of Hodgkin's Disease

Previous Serial Number: NCI-958(c)

Principal Investigator: Ralph E. Johnson, M.D.

Other Investigators: Frederick L. Faw, M.S. and Dwight W. Glenn, M.D.

Cooperating Units: None

Man Years:

Total:	4/2
Professional:	4/2

Project Description:

Objectives: This study is directed toward the improved understanding of the natural history of Hodgkin's disease, the epidemiology of the disease, the methods used in the diagnostic evaluation of patients with respect to determining the anatomic extent of disease, and toward the development of improved therapeutic techniques using radiation therapy alone or, when indicated, in combination with other forms of treatment such as chemotherapy.

Patient Material: A total of 184 patients with previously untreated Hodgkin's disease have been entered into protocol studies since the initiation of this program in January, 1965.

Methods Employed: Information is carefully collected on the clinical course prior to diagnosis including data of epidemiologic interest such as contact with other patients, history of tonsillectomy and previous infections. Extensive diagnostic evaluation is performed prior to therapy to both guide the treatment decision-making as well as to improve our understanding of the natural history of the disease. Various treatment techniques have been prospectively examined by careful controlled clinical trial using radiation therapy alone and in combination with chemotherapy. The clinical course and prognosis have been correlated with a multiplicity of clinical and laboratory parameters of potential value as determinants for the selection of individually optimal treatment.

Major Findings: Many of the findings have been exhaustively described in the publications listed below. The results of this study have permitted

conclusions as to significantly improved methods for the management of patients with Hodgkin's disease and unexcelled therapeutic results and long term survival rates have been achieved.

Proposed Course: Continued long-term observation of surviving patients for evidence of delayed recurrence of disease and/or complications secondary to treatment.

Honors and Awards: None

Publications:

ARTICLES PUBLISHED IN A BOOK

Johnson, R.E.: Total Nodal Irradiation of Hodgkin's Disease: Technical and Clinical Aspects. (In) Textbook of Radiotherapy, Edit. by G. Fletcher, 1972.

Johnson, R.E.: The Curative Radiotherapy of Lymphomas. (In) Recent Results in Cancer Research, Springer-Verlag, New York, 1971.

ARTICLES PUBLISHED IN A PERIODICAL

Faw, F., Johnson, R.E., Glenn, D.W., and Warren, C.A.: A standard set of "individualized" compensating filters for mantle-field radiotherapy of Hodgkin's disease. Amer. J. Roent. 111: 376, 1971

Johnson, R.E.: Is Staging Laparotomy Routinely Indicated in Hodgkin's Disease? Ann. Int. Med. 75: 459, 1971

Johnson, R.E., Glover, M.G., and Marshall, S.K.: Results of Radiation Therapy and Implications for the Clinical Staging of Hodgkin's Disease. Cancer Research 31: 1834, 1971.

Wilson, J.F., Marsa, G.W., and Johnson, R.E.: Herpes Zoster in Hodgkin's Disease: Clinical, Histologic, and Immunologic Correlations. Cancer 29: 189, 1972.

Kagan, A.R., Hafermann, M., Hamilton, M., Pier ce, R., and Johnson, R.E.: Etiology, Diagnosis, and Management of Pericardial Effusion after Irradiation. Radiologia, Clinica, et Biologica 41: 171, 1972.

Johnson, R.E.: Radiation Therapy in Hodgkin's Disease: Total Nodal Irradiation. J.A.M.A., 1972.

Johnson, S.K., and Johnson, R.E.: Does Tonsillectomy Predispose Towards Hodgkin's Disease: A Study of Patients Having Matched Sibling Controls.

Glenn, D.W., and Johnson, R.E.: Male Gonadal Shielding During Cobalt Teletherapy. Radiology(In Press).

Serial No. NCI-963
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Effects of Immunologic Manipulation on Radiation Carcinogenesis and Radiation Response of Tumors

Previous Serial Number: Same

Principal Investigator: Thomas C. Pomeroy, M.D.

Other Investigators: Walter G. Hardy

Cooperating Units: None

Man Years:

Total:	4/4
Professional:	1/4
Other:	3/4

Project Description:

Objectives: To study the effects of immunologic manipulation in the incidence and time of appearance of radiation induced tumors and on the radiation response of tumors.

Methods Employed: Total body radiation of mice with cyclic immunosuppression and in vitro radiation of mouse kidney cells with reintroduction of cells intravenously in cyclically immunosuppressed mice are the methods utilized to evaluate the alterations in types of tumors and time of development of radiation induced benign and malignant neoplasms. Parallel control groups are expected to reveal differences in "spontaneous" tumor production.

Previous studies have shown variations in tumor responsiveness to radiation by non-specific immunologic suppression and enhancement. Presently histopathologic description and correlation of the attendant tissue reactions is being continued.

Major Findings: Immunosuppression delays onset of lymphomas in both control and irradiated mice. In contrast, tumor development other than lymphomas is accelerated in both control and irradiated mice with a greater variety of tumors in the irradiated mice. Despite increased tumor production, cyclic immunosuppression has increased survival rates significantly in both control and irradiated mice. A few adenocarcinomas have developed after in vitro radiation of kidney cells. Histopathologic

changes resemble those of graft rejection where associated with either immunologic enhancement or recovery from immunologic suppression.

Proposed Course: Continue analysis of tumor development and histologic tumor designation. Expand studies to evaluate effects of cyclic immunosuppression in chemical carcinogenesis. Continue efforts to produce transplantable adenocarcinoma of kidney from in vitro irradiation of isologous kidney cells in mice subjected to cyclic immunosuppression.

Honors and Awards: None

Publications:

Thomas C. Pomeroy, and Walter G. Hardy: Enhancement of Radiation Carcinogenesis in Mice by Cyclic Immunosuppression With Cortisone Acetate. Proc. Amer. Assoc. Cancer Res. 13:25, 1972.

Serial No. NCI-964 (c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Study of Cancer of the Upper Air Passages and the Evaluation of Combined Treatment Approaches.

Previous Serial Number: Same

Principal Investigator: Thomas C. Pomeroy, M.D.

Other Investigators: Joseph Belladonna, M.D., Frederick Gibbs, M.D., Walter G. Hardy, and Ralph E. Johnson, M.D.

Cooperating Units: National Institute of Neurological Diseases and Stroke, Surgical Neurology Branch

Man Years:

Total:	3/4
Professional:	2/4
Other:	1/4

Project Description:

Objectives: To evaluate the comparative toxicity and usefulness of various chemotherapeutic agents as adjuvants to radiation therapy in patients with malignant tumors of the mouth and throat.

Laboratory investigations with animal tumor systems will attempt to elucidate additive or synergistic responses and/or toxicity of various combinations of modalities and sequences of administration.

Patient Material: Patients with post-surgical and post irradiation recurrences and advanced inoperable squamous cell carcinomas of mouth and throat are accepted for study.

Methods Employed: (1) Post-surgical and post radiation recurrences of squamous cell carcinomas of mouth and throat are evaluated for toxicity and tumor response to selected agents for which evidence exists suggesting a response of this type of malignancy (e.g. Bleomycin, Methotrexate, and Adriamycin. (2) Active drugs and combinations of active drugs will be added to radiation therapy in post-surgical recurrent and advanced inoperable epidermoid cancers of the mouth and throat. (3) Laboratory studies combine radiation of solid implants with systemic chemotherapy in varied sequences and time-dose relationships, and evaluation of

various combined schedules of Bleomycin, Adriamycin and Methotrexate in the L 1210 Leukemia model system.

Major Findings: (1) Limited observations indicate guarded optimism for the usefulness of Bleomycin in management of squamous cell carcinoma. In particular its lack of hematologic toxicity enhances its use with other types of agents. Addition of MTX has shown an apparent synergistic action in two patients with complete clinical disappearance in both and histopathologic disappearance in one. A third patient has also shown a very striking additive effect. Further studies are required to fully document these observations. (2) Adjuvant chemotherapy and radiation therapy of post-surgical and advanced inoperable cases have been initiated and have shown uniformly complete clinical control within the irradiated volume for periods up to one year in nine patients. Four of these patients have developed distant metastasis without local recurrence. Two additional patients are now receiving treatment. These results to date indicate little optimism for long-term control in this type of relatively hopeless clinical situation, unless more aggressive chemotherapy can control the disseminated disease or earlier stages of disease can be treated. (3) Tumor model systems in mice have shown additive effects of MTX and radiation, Bleomycin and radiation and the best tumor control with MTX, Bleomycin and radiation and the best tumor control with MTX, Bleomycin, and radiation. Bleomycin combined with hyperbaric oxygen showed a greatly increased pulmonary toxicity indicating the necessity for extreme caution in any clinical trials combining these two modalities.Various combinations of Bleomycin, MTX, and Adriamycin are now being tested with L 1210 Leukemia Model System, with particular consideration of preoperative combined therapy in earlier stages of disease and application of multiple drug therapy to control disseminated tumor.

Proposed Course: To continue and expand these studies.

Honors and Awards: None

Publications: None

Serial No. NCI-965 (c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Study of Hand-Schuller-Christian Disease and the Role for Radiation Therapy of the Hypothalamic-Pituitary Lesion

Previous Serial Number: Same

Principal Investigator: Thomas C. Pomeroy, M.D.

Other Investigators: Mortimer Lipsett, M.D.; Griff Ross, M.D.; and Glenn Braunstein, M.D.

Cooperating Units: National Institute of Child Health and Development-Reproduction Research

Man Years:

Total:	1/6
Professional:	1/12
Other:	1/12

Project Description:

Objectives: To determine the results of radiation therapy directed to the hypothalamic-pituitary region on the growth hormone production and the severity and progression of diabetes insipidus in patients with Hand-Schuller-Christian disease.

Patient Material: Fifteen patients in the pre-pubertal age range with biopsy proven Hand-Schuller-Christian disease with diabetes insipidus, with or without clinical growth retardation, but without generalized disease will be acceded to the study.

Methods Employed: Extensive clinical and laboratory evaluation of each patient will precede radiation therapy. This will include growth hormone assays and water loading tests. Radiation therapy (1200 rads in two weeks) will be followed by serial determinations of growth hormone production and evaluation of any changes in growth patterns and severity of diabetes insipidus.

Major Findings: Results in the first case treated, though preliminary, have shown an increase in the growth hormone level after radiation, but without increase in rate of growth. A second case has shown increased growth rate but no significant change in growth hormone level.

Proposed Course: Continue accession of the planned number of patients.

Honors and Awards: None

Publications: None

Serial No. NCI-975(c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Study of Cancer of the Lung

Previous Serial Number: None

Principal Investigator: Kent B. Lamoureux, M.D.

Other Investigators: Ralph E. Johnson, M.D.

Cooperating Units: Laboratory of Pathology, NCI and Departments of Nuclear Medicine and Diagnostic Radiology, Clinical Center, NIH

Man Years:

Total: 3/2

Professional: 3/2

Project Description:

Objectives: This study is directed toward improving understanding of the epidemiology and natural history of cancer of the lung, developing methods which will facilitate earlier diagnosis of the disease, and achieving improved survival rates through exploration of therapeutic approaches which combine the various available modalities of treatment.

Patient Material: The initial pilot studies have been instituted during the past few months and a limited number of patients have been accession ed into protocol investigations. A rapid expansion of this program is anticipated during the coming year.

Methods Employed: A linear array program is currently being developed which will coordinate efforts from multiple disciplines and, on the basis of which, target areas will be identified for investigation utilizing both intramural and extramural resources. The present active studies are limited to therapeutic efforts to improve the survival of patients with unresectable disease by means of combined treatment with radical radiation therapy and intensive systemic chemotherapy. These therapeutic trials are simultaneously generating information on the natural history of lung cancer, the reliability of various diagnostic procedures, and other aspects of lung cancer which will be requisite for the rational expansion of studies in ancillary areas of investigation.

Major Findings: There have been no major findings to date.

Proposed Course: This will be a long-term study which will continue for an indefinite period of time.

Honors and Awards: None

Publications: None

Serial No. NCI-976(c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Study of Chronic Lymphocytic Leukemia

Previous Serial Number: NCI-6054(c)

Principal Investigator: Ralph E. Johnson, M.D.

Other Investigators: Joseph Belladonna, M.D.

Cooperating Units: None

Man Years:

Total: 1/4

Professional: 1/4

Project Description:

Objectives: This study is directed toward the improved understanding of the natural history of chronic lymphocytic leukemia, the delineation of the factors related to the disease which adversely affect the patient such as secondary effects on the immune mechanism, the establishment of criteria for the initiation of treatment in patients with relatively asymptomatic disease on a morphologic basis, and finally toward the development of improved techniques for therapeutic management using single and combined modalities of treatment.

Patient Material: Patients with previously untreated chronic lymphocytic leukemia are studied longitudinally for the purpose of defining the natural history of the disease. Various factors are serially surveyed such as the integrity of the immune mechanisms both prior to and following treatment. Approximately thirty patients have been studied to date.

Methods Employed: In addition to diagnostic and immunologic studies as mentioned above, attempts are being made to improve the effectiveness of therapeutic management using the modalities of radiation therapy and chemotherapy both individually and in combination.

Major Findings: The natural history of chronic lymphocytic leukemia is being prospectively defined, and factors are being ennumerated which serve as determinants for segregating patients with indolent or "benign" forms of the disease from those with active or "aggressive" phases of chronic lymphocytic leukemia. The therapeutic trials have served to

demonstrate that more complete remissions than anticipated with other forms of treatment can be regularly achieved with total body irradiation. At present, attempts are being made to improve these remission rates with combined form of treatment.

Proposed Course: The natural history of chronic lymphocytic leukemia demands long-term study and observation of patients will continue indefinitely in addition to the accession of new patients into the program.

Honors and Awards: None

Publications:

ARTICLES PUBLISHED IN A BOOK:

Johnson, R.E.: Treatment of Chronic Lymphocytic Leukemia. (In) Recent Results in Cancer Research. Publ. by Springer-Verlag, New York, 1971

ARTICLES PUBLISHED IN A PERIODICAL

Johnson, R.E.: Total Body Irradiation of Chronic Lymphocytic Leukemia: Incidence and Duration of Remission: Cancer 25: 523, 1970.

Wilson, J.F., and Johnson, R.E.: Splenic Irradiation Following Chemotherapy in Chronic Myelogenous Leukemia, Radiology 101: 657, 1971.

Serial No. NCI-6054(c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Study of Non-Hodgkin's Lymphomas

Previous Serial Number: Same

Principal Investigator: Ralph E. Johnson, M.D.

Other Investigators: None

Cooperating Units: Laboratory of Pathology, NCI and the Departments of Nuclear Medicine, Diagnostic Radiology, and Hematology of the Clinical Center, NIH; Medicine Branch, NCI

Man Years:

Total:	2/2
Professional:	2/2

Project Description:

Objectives: The study is directed toward the improved understanding of the epidemiology and natural history of the non-Hodgkin's lymphomas, the methods used for the diagnostic evaluation of patients, and the development of improved therapeutic techniques using radiation therapy alone or, when indicated, in combination with other forms of treatment such as chemotherapy.

Patient Material: This is a collaborative effort involving various clinical groups at the NIH and includes both comparative and combined therapeutic trials, into which approximately one hundred patients have been accessioned to date.

Methods Employed: Patients are extensively evaluated with the collection of detailed information on the clinical course prior to diagnosis including data of epidemiologic interest. An exhaustive diagnostic study of all patients is providing critical information on the natural history of these diseases and is serving to create a rational background against which the currently accepted forms of treatment can be meaningfully interpreted. Various treatments, whether with single modalities or combined techniques, are being examined in controlled, prospective fashion and the clinical course and prognosis are being correlated with multiple parameters of potential value as determinants for the future selection of optimal treatment for individual patients.

Major Findings: Many of the findings have been described in the publications listed below. The results of this study have permitted conclusions as to improved methods for the management of patients with non-Hodgkin's lymphomas and greatly improved therapeutic results have been achieved, particularly for the significant fraction of cases who have relatively advanced disease at the time of diagnosis.

Proposed Course: Continued accession of patients and long-term observation for patients already under study.

Honors and Awards: None

Publications:

ARTICLES PUBLISHED IN A BOOK

Johnson, R.E.: The Curative Radiotherapy of Lymphomas. (In) Recent Results in Cancer Research, Springer-Verlag, New York, 1971.

ARTICLES PUBLISHED IN A PERIODICAL

Johnson, R.E.: Remission Induction and Remission Duration with Primary Radiotherapy in Advanced Lymphosarcoma. Cancer(In Press, 1972)

Johnson, R.E.: Management of Generalized Lymphosarcoma with Radiation Therapy. Amer. J. Roentgen.(In Press, 1972).

Johnson, R.E., Chretien, P., and O'Conor, G.T.: Influence of Laparotomy Findings on Treatment Planning in Non-Hodgkin's Lymphomas(In Press).

Serial No. NCI-6502(c)
1. Radiation Branch
2. Radiation Therapy Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Study of Ewing's Sarcoma as a Model System in Experimental Therapeutics for Adjuvant Chemotherapy

Previous Serial Number: Same

Principal Investigator: Ralph E. Johnson, M.D.

Other Investigators: Thomas C. Pomeroy, M.D.

Cooperating Units: None

Man Years:

Total: 1/4

Professional: 1/4

Project Description:

Objectives: Ewing's sarcoma, while relatively uncommon with respect to incidence in the general population, has a natural history and biologic behavior which provides an excellent model system for the study of multi-system treatment in man. The marked propensity for patients to develop early metastatic spread renders treatment directed only to the primary tumor inadequate to achieve cure in the great majority of cases. This affords an excellent situation to examine the merit of the adjuvant therapy concept and, to this end, intensive systemic chemotherapy is being evaluated as an adjuvant to treatment of the primary tumor with either surgery or radiation therapy.

Patient Material: Patients with a diagnosis of Ewing's sarcoma have been admitted for either primary treatment or, in selected instances, for secondary treatment when suitable for Phase II chemotherapy trials. To date, a total of 35 patients with clinically localized disease have been admitted plus a somewhat smaller number with either metastatic or recurrent disease following primary treatment.

Methods Employed: In the case of clinically localized disease, intensive systemic chemotherapy has been combined with primary treatment using radiation therapy or surgery. Irradiation of the central nervous system has been evaluated to eradicate possible metastatic disease protected from drug effect by the blood-brain barrier. And a panel of nationally recognized bone tumor pathologists have reviewed the biopsies.

Major Findings: A 2-year disease free survival rate of approximately 50% has increased by several fold the results achieved with primary treatment confined to the primary tumor. This study has served to convincingly demonstrate the important potential role for adjuvant chemotherapy in at least one malignant with a high propensity for early metastatic spread prior to diagnosis.

Proposed Course: With the addition of a relatively small number of additional patients to the study to evaluate a hopefully improved scheme of adjuvant chemotherapy, accession of patients will be terminated and observation on treated patients continued indefinitely for long term results.

Honors and Awards: None

Publications:

ARTICLES PUBLISHED IN A BOOK: None

ARTICLES PUBLISHED IN A PERIODICAL:

Marsa, G.W., and Johnson, R.E.: Altered Pattern of Metastasis Following Treatment of Ewing's Sarcoma with Radiotherapy and Adjuvant Chemotherapy. Cancer 27: 1051, 1971.

Pomeroy, T.C., and Johnson, R.E.: Integrated Therapy for Ewing's Sarcoma. Proc. Amer. Assoc. Cancer Res., Cancer Res. 12: 80, 1971.

Johnson, R.E., and Pomeroy, T.C.: Integrated Therapy for Ewing's Sarcoma. Amer. J. Roentgen. 114: 532, 1972.

Oldham, R.K., and Pomeroy, T.C.: Treatment of Ewing's Sarcoma with Adriamycin. Cancer Chemo. Rep.(In Press).

Serial No. NCI-684
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Non-Clinical Service Irradiation

Previous Serial Number: Same

Principal Investigator: Robert W. Swain, B.S.

Other Investigators: Thomas L. Bryant, and Gilbert M. Bowie

Cooperating Units: None

Man Years:

Total:	2/4
Professional:	1/4
Other:	1/4

Project Description:

Objectives: To provide facilities for the performance of irradiation of biological materials and animals as required for research studies by investigators outside of the Radiation Branch. This includes consultation as to methods and types of irradiation and the preparation of material.

Methods Employed: The three Mev Van de Graaff generator for high energy electrons and x-rays and a 250 Kvcp x-ray generator are provided and maintained in proper operating condition.

Major Findings: The attached tables indicate the organization for which irradiations were performed and the number of exposures.

Proposed Course: To continue

Honors and Awards: None

Publications: None except as indicated by the use of these facilities in publications from other laboratories.

Services Provided Using the 250 KVCP
Double Header Westinghouse Quadrocondex
From April 1, 1971 to April 1, 1972

Organization	Number of Exposures
A-A&R	164
A-LEP	12
A-LPB	60
BS-LVI	33
C-BGY	301
C-CT	108
C-D	8
C-GL&C	116
C-HTCB	147
C-I	336
C-LBGY	178
C-LCB	74
C-LP	58
C-LPHY	862
C-M	131
C-MET	120
C-PA	6
C-R	39
C-SURG	60
C-VB	39
C-VLL	84
CC-CP	1
CH-RR	2
D-LB	11
D-LM	36
D-OMS	60
I-LBV	13
I-LCI	55
I-LI	354
I-LM	82
I-LMI	226
I-LPD	8
I-LVD	95
*MICRO	37
**Other	375
Total	4291

* Exposures for Microbiological Associates were performed on materials for projects under contract to the National Cancer Institute.

** Institute and Branch not identified.

Materials irradiated using the 250 KVCP Double Header Westinghouse Quadrocondex included cells, tissue cultures, blood, mice, rats, hamsters, chickens, and guinea pigs. Exposures ranged from 100 R to 15000 R.

Services Provided Using the
FD Van de Graaff Generator
From April 1, 1971 to April 1, 1972

Organization	Number of Exposures
H-LTD	5
R-LA	25
*MICRO	6
Navy	7
Walter Reed	6
	49

* Exposures for Microbiological Associates were performed on materials for projects under contract to the National Cancer Institute.

Materials irradiated with electrons were animal food, albumin, sucrose, dura heart valves, cells, and permeable membranes. Total doses from electrons ranged from 10^4 to 10^7 rads.

Serial No. NCI-947
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Development, Fabrication and Maintenance of Specialized Equipment for Radiation Branch, Laboratory of Physiology, and Several Other Laboratories in NCI

Previous Serial Number: Same

Principal Investigators: Nathan F. Coffey and Edward J. LeBrun and Ralph C. Whittaker

Man Years:

Total:	4.1
Professional:	2.1
Others:	2.0

Project Description:

Objectives: After discussion with the senior scientists in the NCI located in Building 10, designs and fabricates instruments that are not available on the open market. Modifications of existing equipment are also performed to increase its versatility, accuracy, and requirements. In addition, servicing and repairing heavy equipment, such as Van de Graaff generators, x-ray equipment, and mechanized automatic recording devices are undertaken. Glass blowing services, including designing, fabrication, and repair, are also provided.

MACHINE SHOP

The following percentages indicate proportion of major work performed by Machine Shop for various NCI laboratories:

	% OF TIME	HOURS	NO. OF JOBS
C-R	55	1787	63
C-LPHY	17	553	45
C-I	10	325	36
C-PA	5	163	39
C-M	5	163	29
C-BGY	4	130	32
C-MET	2	65	14
C-LCBGY	2	65	17
		3251	275

Approximately 170 hours consultation with various investigators.

GLASS SHOP

	% OF TIME	HOURS	NO. OF JOBS
C-LPHY	50	1650	70
C-I	19	550	30
C-LBGY	20	692	29
C-PATH	5	40	4
C-PHARM	5	32	4
C-R	-1	8	2
	100	2962	139

Approximately 100 hours consultation with various investigators.

ELECTRONICS SHOP

	% OF TIME	HOURS	NO. OF JOBS
C-R	69		4
C-LPHY	29		29
C-I	1		3
R-BEI	less than 1		1
ODA A PS	less than 1		2
C-EA	less than 1		1
		TOTAL	40

Serial No. NCI-966 (c)
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Tumor Diagnosis With Microwaves

Previous Serial Number: None

Principal Investigator: Dwight W. Glenn, M.S.

Other Investigators: Thomas C. Pomeroy, M.D.

Cooperating Units: None

Man Years:

Total: 1/12
Professional: 1/12

Project Description:

Objectives: To determine the feasibility of using microwaves to supplement or replace mammography, ultasonics, thermography, or other means in localizing tumors.

Methods Employed: Kearfott Division of Singer Company has proposed to fabricate suitable transmitters and receivers for diagnostic purposes. In-vitro evaluation will be made with phantoms followed by in-vivo evaluation with small animals here at NCI.

Proposed Course: Now waiting for proposal from Kearfott Division on estimated cost of production of devices. If reasonable from cost standpoint, we will request funding for this project.

Honors and Awards: None

Publications: None

Serial No. NCI-967
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Measurement of "W" for 14 Mev Neutrons

Previous Serial Number: None

Principal Investigator: Dwight W. Glenn

Other Investigators: Nathan Coffey

Cooperating Units: None

Man Years:

Total: 3/4
Professional: 3/4

Project Description:

Objectives: To determine the value of "W" for 14 Mev Neutrons in order to convert ionization measurements to absorbed dose measurements for possible neutron radiotherapy and/or radiobiological applications.

Methods Employed: Tissue-equivalent plastic, fabricated into ionization chambers and calorimeters, will give a direct comparison between ionization and energy absorption. Standardization will be made with photon irradiation prior to irradiation with the neutron beam from the 14 Mev Kaman generator and other available neutron beams.

Major Findings: Since the measurement system is not yet operational, the only reportable findings relate to failure of components.

Proposed Course: The system should be operational by July 1, 1972. Data collection will be initiated using the cobalt beam. If successful, neutron data collection will follow.

Honors and Awards: None

Publications: None

Serial No. NCI-968 (c)
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Gonadal Shielding

Previous Serial Number: None

Principal Investigator: Dwight W. Glenn, M.S.

Other Investigators: Ralph E. Johnson, M.D.

Cooperating Units: None

Man Years:

Total: 1/12
Professional: 1/12

Project Description:

Objectives: To determine the most efficient shielding technique for male gonads during irradiation of adjacent volumes.

Methods Employed: Dosimetric evaluation by lithium fluoride and ionization chambers when utilizing various shields around and/or over the gonads.

Major Findings: The gonadal dose is minimized by utilizing a two component shield--one placed in the beam over the patient and a lead cone placed around the gonads.

Proposed Course: Completed.

Honors and Awards: None

Publications: Submitted as Technical Note in Radiology.

Serial No. NCI-970 (c)
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Patient Positioning Television System

Previous Serial Number: None

Principal Investigator: Albert P. Wheatley, SPS, ET

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1/6
Professional: 0

Project Description:

Objectives: The objectives of this project is to provide a closed circuit television system which may be used to insure, to a high degree of accuracy, the placement of a patient in a radiotherapy beam, and to provide visual graphic proof of that placement on each successive treatment.

Methods Employed: A line diagram of a CCTV system was devised and the components for that system were placed on order from Video Engineering Inc., of Washington, D.C. The equipment was installed by Video Engineering and acceptance tests were made and completed during January, 1972.

Further tests made showed that the television monitor used to photograph the image of the patient was not linear and that no round screen picture tube can be used for that purpose. Subsequently, a flat faced, highly linear, kinescope recording monitor was ordered from Conrac, Inc. of Covina, California.

At the present time, the system is usable with the error induced from a rounded screen monitor and is awaiting the arrival of the new monitor.

Upon completion of the project, the treatment technician will be able to take pictures of the television image which will show exactly where in relation to the therapy device used that the patient is placed, both initially and on each subsequent treatment.

Major Findings: The face of television picture tube must be flat in order to photograph it with sufficient accuracy to project that photograph back onto the television image and achieve maximum similarity of images.

Proposed Course: See above.

Honors and Awards: None

Publications: None

Serial No. NCI-971 (c)
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Isodose Plotter

Previous Serial Number: None

Principle Investigators: Albert P. Wheatley; Frederick L. Faw, M.S.

Other Investigators: Robert W. Swain, B.S.

Cooperating Units: None

Man Years:

Total: 1/3
Professional: 1/4

Project Description:

Objectives: To develop an Isodose Plotter package which is controlled by a mini-computer to measure the relative amounts of radiation in a controlled field of such ionizing radiation.

Methods Employed: Using an old plotter and replacing the servo-motors with stepping-motors, a basic design of a three dimensional plotter was made. From this design, a proto-type plotter was built and the necessary associated electronics were designed and built.

Major Findings: Initially, these electronics provided two distinct analog channels to the computer and the program took care of finding the ratios of these signals. Since there was a time difference between the reading of these two channels, significant noise was observed at the output of the pulsed linear accelerator. In order to minimize the noise to that level which is really in the Linac, the plotter electronics were modified to include a ratio circuit and the output of that circuit is fed to the computer on a single analog channel.

Proposed Course: Since the present plotter was intended to be a proto-type and has been improved both mechanically and electronically since its initial assembly, we plan to build a new plotter and increase its size so as to allow the scanning of the largest possible fields.

Honors and Awards: None

Publications: None

Serial No. NCI-972 (c)
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: High Output Linear Accelerator

Previous Serial Number: None

Principal Investigator: Robert W. Swain, B.S.

Other Investigators: Dwight W. Glenn, M.S.; Frederick L. Faw, M.S.; and Albert P. Wheatley

Cooperating Units: None

Man Years:

Total: 1/3
Professional: 1/3

Project Description:

Objectives:

1. To assure reliable dosimetry for patient treatment.
2. To be able to provide electron irradiations of sufficiently high dose rate to replace the electron capability of the 3 Mev Van de Graaff generator.

Methods Employed:

1. Routine requirements:
   a. Start-up and adjustments daily.
   b. Calibration once each week.
   c. Check on symmetry once each week.
   d. Check on dose distribution once each month.
   e. Perform routine maintenance as suggested by manufacturer.
2. Design and install dual ionization chamber for x-rays.
3. Complete modifications for obtaining high dose electrons.
4. Determine electron dose distributions for normal and high dose rate electrons.

Major Findings to Date:

1. Unit was accepted from the manufacturer on February 2, 1971.
2. From date of acceptance to August 31, 1971, unit was checked for reliability, output was calibrated, and dose distributions were determined.

3. Treatments were begun on August 31, 1971, and continued through September 13, 1971.
4. During dosimetry check on September 14, 1971, a vacuum failure occurred in the vacuum envelope. A new vacuum envelope was installed. During the seasoning of the new vacuum envelope a second vacuum failure occurred which let water enter the accelerating wave guide. After some attempt to repair on the site, the wave guide was sent to the factory, repaired, and returned.
5. The company has had one to three engineers here continuously since the waveguide has been returned. The major problem appears to be the beam not entering the center of the vacuum envelope in which the beam is bent. It has been determined that the vacuum envelope was bent so that the input and output ends were out of line by approximately 1/4 inch. It could well be that this has been the cause of difficulty since the middle of December.
6. At this time, it is difficult to predict when the unit will be available for treatment.

Proposed Course: See Methods Employed.

Honors and Awards: None

Publications: None

Serial No. NCI-973
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cesium-137 Irradiator

Previous Serial Number: None

Principal Investigator: Robert W. Swain, B.S.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1/4
Professional: 1/4

Project Description:

Objectives: The objective of this project is to provide a relatively high output irradiator for the radiation of small animals and biological materials which will provide reliable dosimetry and be simple in operation.

Methods Employed: Contract was let to Lockheed-Georgia on June 28, 1969. Partial shipment and installation has been made. Progress has been delayed by the closing down of the Nuclear Division of the company and by changes in personnel including project engineers.

About the end of February, a proposal was received to complete the project at an additional cost of $71,773. This cost included the manufacturing of a new source train loaded with non-radioactive cesium chloride, the testing of the sources according to the proposed classification of sources by USASI, committee N5.4, and the design and installation of a mechanical cushioning device.

A site visit was made by the Project Officer, a representative from Radiation Safety, the Contract Officer, and a representative from DHEW legal department on April 14. Although no commitments were made during the visit, it has been decided to terminate the contract with Lockheed. The only possible legal recourse would be to prove that Lockheed had not put forth their "best effort" which would be very difficult to prove.

A draft of a proposal to complete the unit has been prepared by Dr. B. E. Leonard of the Institute for Resource Management. Other companies are being asked to survey the situation and to make proposals.

Major Findings: See above.

Proposed Course: See above.

Honors and Awards: None

Publications: None

Serial No. NCI-974
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Removal of Van de Graaff Generator

Previous Serial Number: None

Principal Investigator: Robert W. Swain, B.S.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1/12
Professional: 1/12

Project Description:

Objectives: Remove 3 Mev Van de Graaff generator and auxilliary components from the Clinical Center.

Methods Employed: Sections of the generator located in Building 10, Room B3B54 will be removed personally. The removal of the remainder will require supervision. The Plant Engineering Branch has been taking care of plans for dismantling and the contract for removal with my consultation.

A contract was let for bids for either removing the unit as a usable piece of equipment or for scrapping it. The Lawrence Radiation Laboratory had indicated a need for it and was going to pay the difference in the cost. The bids were received at $50,000 and $30,000, respectively. LRL does not want it now so the unit will be scrapped.

It is estimated that work on the project will begin the end of May or the first part of June.

Major Findings: See above.

Proposed Course: See above.

Honors and Awards: None

Publications: None

Serial No. NCI-6050 (c)
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Computer Applications in Radiation Therapy

Previous Serial Number: Same

Principal Investigator: Frederick L. Faw, M.S.

Other Investigators: Dwight W. Glenn, M.S.; Robert W. Swain, B.S.; and Albert Wheatley

Cooperating Units: None

Man Years:
Total: 9/12
Professional: 9/12

Project Description:

Objectives: To develop techniques for the utilization of computers in increasing the speed and accuracy with which complicated dosimetric calculations can be performed.

Methods Employed: An "in-house" computer located in the Radiation Branch has been programmed for use in clinical radiation therapy planning and to measure dose distributions, plotting the resultant isodose curves. Short programs are run on the Central Computer here at NIH.

Major Findings: Isodose curves are now available for the linear accelerator. The isodose plotter is routinely used to check the output of the linear accelerator.

A patient data retrieval system was initiated and preliminary usage indicated that time for data analysis was greatly decreased and accuracy was increased.

Proposed Course: Eventually, it is anticipated that all treatment planning and treatment administration will utilize computer control in the Radiation Branch.

Honors and Awards: None

Publications: None

Serial No. NCI-969 (c)
1. Radiation Branch
2. Radiation Physics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Large Field Dosimetry

Previous Serial Number: None

Principal Investigator: Frederick L. Faw, M.S.

Other Investigators: Dwight W. Glenn, M.S.

Cooperating Units: None

Man Years:

Total: 3/12
Professional: 3/12

Project Description:

Objectives: To develop a technique by which the dose distribution in patients when the field is larger than the part of the body being treated can be estimated.

Methods Employed: Dose distributions in phantoms are determined using an isodose plotter and thermoluminescent dosimeters. These data are compared with published data and in vivo doses from patients.

Major Findings: When the field is larger than the patient or part of the patient being treated, the dose distribution is determined by the size of the patient rather than the size of the field.

Proposed Course: To be completed during the coming year.

Honors and Awards: None

Publications: None

ANNUAL REPORT SUMMARY

SURGERY BRANCH

NATIONAL CANCER INSTITUTE

April 1, 1971 - March 31, 1972

The Surgery Branch has continued the dual responsibility of providing surgical consultative and operative assistance to the multi-disciplined intramural clinical units of the National Institutes of Health while developing its own laboratory and clinical research programs. Approximately 60% of the Surgery Branch staff's activities are consumed by clinical responsibilities. During the past 12 months, 804 individually submitted surgical consultations were received, in addition to supervising 704 requests for consultative services from extramural consultant sources. These figures include only the original consultative requests and are not inclusive of the tremendously larger total number of visits that were repetitively made to follow up the surgical consultative recommendations or surgery itself. Surgical procedures were performed upon 680 patients during the past year, of which 228 were performed as part of the specific clinical research program of the Surgery Branch itself.

The ongoing clinical investigative program of the Branch can be categorized into four areas of chief interest: Cancer of the pelvic structures (cervix), head and neck area, soft tissue and bone sarcomas, and melanomas. Cervix: Extended surgical procedures are the only effective treatment modalities for patients with radiorecurrent cervical cancer and have been demonstrated to be effective alternatives for the management of selected patients with primary cancer of the cervix. Although the Branch is no longer recruiting patients with cancer of the cervix, significant numbers of patients with radiorecurrent disease are still being referred here. Using extended surgical procedures of pelvic exenteration, an actuarial 5-year survival of 67.6% was obtained in 89 patients with locally advanced primary cervical cancer and 39.1% in 110 patients with recurrent disease. Radical hysterectomy performed in 84 patients has resulted in an 86% survival. Rehabilitative procedures such as vaginoplasties continue to be successfully utilized in selected patients. Head and Neck: In the treatment of head and neck cancer the main reason for failure is recurrent disease in the local area. In line with recent experimental evidence, which indicates that the few cells remaining after the main bulk of tumor has been removed proliferate at an increased rate and are more sensitive to chemotherapy agents, a protocol is in progress to evaluate the clinical response. In the first 10 patients treated with high dose Methotrexate preoperatively, 8 have shown a marked reduction in the size of the tumor. Of the 8 patients placed on long-term (2 years) intermittent therapy postoperatively with the same drug there has been no evidence of recurrent disease. The continued interest in paranasal sinus cancer is satisfied by the 60% survival now obtained in patients who underwent the NIH developed craniofacial exenteration for disease which involves the ethmoid sinuses. The use of preoperative irradiation as an adjuvant to disease of this and other anatomical areas of head and neck has not proven to be of value by increasing survival but has been of extreme significance in decreasing morbidity. A cooperative adjuvant chemotherapy program is now underway in an attempt to offer

further therapeutic modalities to patients with advanced head and neck cancer. There is a continued cooperative investigation with the NHLI studying the mechanisms of taste and smell and the compensatory alterations in these functions following head and neck surgery. After removal of the olfactory nerve and primary olfactory epithelium in 25 patients, they were still able to detect odors using accessory areas of olfaction in the oropharynx. In 35 patients after total laryngectomy in which the primary olfactory area was not surgically disturbed all patients lost their ability to detect odors in a normal fashion. Utilizing accessory areas in the oropharynx they were able to detect odors but at a higher threshold level. It appears that surgical interference with sensory nerves to the larynx at the time of its removal may significantly alter olfactory acuity through some complex feedback mechanism or by interfering with a diffuse anatomical projection system from pharynx and/or larynx to the olfactory brain. Sarcomas: Soft tissue and particularly bone sarcomas remain an enigma to the cancer therapists. The Branch experience with a limited number of each of the particular sarcomas indicates that the 5-year survival following amputative surgery, with and without adjuvant therapy of several modes, is approximately 12% with osteogenic sarcomas and 49% with chondrosarcomas. The usual fibrosarcoma, rhabdomyosarcoma, and liposarcoma patient has a survival of approximately 52%. It has been shown that hemipelvectomy can be safely performed and can be curative in approximately 50% of instances. In several animal tumor systems, it has been repeatedly demonstrated that a safe level of constant coumadin anticoagulation can be attained through heparin medicated drinking water. Using spontaneously arising and intramuscularly transplanted tumor, anticoagulated animals have a significant reduction in the number who develop metastases and in the amount of metastatic disease occurring in those animals who do develop metastases. The results of this work have now been incorporated in a clinical protocol in which patients undergoing surgery for sarcoma, known to be prone to hematogenous tumor dissemination, are anticoagulated preoperatively and postoperatively maintained indefinitely with a 2 to 3 times increase in their prothrombin time. In the 11 patients who have thus far been entered into this protocol and maintained for months on anticoagulation there has been some increased operative morbidity, but of a nature not significant to interfere with the continuation of the protocol investigation. Melanoma: In achieving local control of the cutaneous lesions, results of standard wide-margin surgical excision are satisfactory with a recurrence rate of less than 7%. Failure to control distant metastatic disease, however, is the primary cause of the overall mortality of 54% in 5 years. In determining what role prophylactic and therapeutic node dissections, anatomic site and histology of the primary lesion, and special host characteristics play in survival, the experience of the Branch with 240 patients is under review. The results of this review may constitute a surgical base line from which adjuvant programs can be designed to further control melanoma metastases.

In conjunction with the Department of Cytopathology a program has been instituted to evaluate the effectiveness of fine needle (gauge 22 or 25) aspiration biopsy in the cytologic diagnosis of solid tumors. This technique has proven to be of extreme value in the Scandinavian countries, and this cooperative study was started to evaluate the technique here using both cytology and hematology stains. Over 200 aspirations have been performed to date of melanoma; squamous carcinoma; lymphoma; adenocarcinoma; breast, thyroid, prostate, liver, intra-abdominal, and pelvic masses; and surface tumors. The incidence of false

negatives is 5% and false positives 0. The technique shows a great deal of promise and in the future should reduce the need for excisional biopsy of breast masses by two-thirds.

Surgical Physiology. Four main areas of physiological investigation are under study: The study of fluid volume and albumin kinetics; capillary permeability studies in states of stress and other trauma; studies on etiology, pathogenesis, and treatment of the syndrome post-traumatic pulmonary insufficiency (congestive atelectasis); and the development of a method of autotransfusion to be used during extensive cancer surgical procedures. Significant developments to date include the following: After major cancer surgery we have demonstrated a loss of 24% of the patient's plasma volume into a noncirculating or nonavailable pool. In addition, some 33% of the total circulating proteins disappear from the circulation during the course of a major surgical procedure. This protein loss from the circulation is not available to the body, as in metabolic measurements of the total body albumin before and after major surgery up to 29% of the total body albumin is in a nonexchangeable area. This sequestration of albumin into a noncirculating or nonexchangeable pool remains in this area for a period of 7-10 days. We were unable to demonstrate a rapid influx of albumin from the extravascular compartment into the vascular system after a major surgical procedure and indeed the albumin in the vascular pool remained between 20 and 30% deficient for a period of at least 24 hours postoperatively. These studies indicated the need for vigorous replacement albumin during and following a major surgical procedure, and we have found that as much as 300 grams must be given to some patients over a 24 hour period. Since the institution of this regimen of management, abnormalities in the patient's physiological functions postoperatively have been much less frequently observed. For the study of capillary permeability we have completed standardization of gel columns and automatic determination of dextran fractions. We now have the capability to study changes in capillary pore size varying from 10,000 to 150,000 molecular weight. In the experimental animal we have reproduced a pathological picture of post-traumatic pulmonary insufficiency in an isolated perfusion lung circuit. Studies are currently underway to evaluate the various causes of factors believed to be associated with this uniformly fatal postoperative complication. Heretofore, the syndrome has been difficult to reproduce experimentally, and the lack of a suitable model has interfered with progress in understanding the disease. We have found in both the dog and baboon that perfusion with blood stored for 21 days and filtered through a standard administration set causes increased pulmonary vascular resistance, decreased lung compliance, decreased oxygen uptake by the lung, pulmonary vascular congestion, and interstitial edema. After identifying the elements in blood which give rise to these changes, we hope to be able to alter homologous blood by centrifugation or filtration in such a manner that the microaggregates are not infused and presumably the pulmonary insult will not occur.

A significant contribution of the Branch was the first demonstration of the common tumor antigen in patients with sarcomas. When assayed with immunofluorescence, cytotoxicity and complement fixation techniques, a specific antibody in sera from all patients with this disease has been found. These results suggest the presence of an associated viral agent in human sarcomas. Indirect evidence for this is a common cross-reacting sarcoma-specific antigen

found in all sarcomas studied. The presence of a filtrable agent was directly demonstrated by the demonstration that cell-free extracts both from sarcoma tumor tissue and concentrated media from tissue cultures of human sarcomas were capable of infecting normal human embryonic cells in tissue culture and inducing the formation of a new sarcoma-specific antigen. These antigenically transformed cells reacted with serum from sarcoma patients, whereas normal uninfected cells did not. The studies which showed that immunologic factors are associated with sarcomas encouraged us to undertake preliminary studies in immunotherapy of this disease. After immunization with autologous tumor antigen and BCG, 9 of 12 sarcoma patients showed an increase in cytotoxic antibodies when their sera was tested against a homologous sarcoma tissue culture line, and 2 had rejective remissions induced by this therapy. Failure to achieve clinical remission in most patients must be viewed in the context of the disease status of the patients being treated. All had diffuse metastases in the presence of which, even with the efficacious chemotherapy regimen, only minimal symptomatic palliation intervals would have been achieved. The use of this therapy as an adjuvant to definitive surgical treatment for the eradication of remaining microscopic foci of tumor would appear to be a more reasonable goal for immunotherapy.

The Branch first demonstrated a common tumor antigen in patients with melanoma, using the technique of tumor cell immunofluorescence. These studies led to an immunotherapy regimen in patients with advanced melanoma and multiple subcutaneous metastases. Eighteen patients had injection of BCG directly into their metastatic lesions. Five have shown some response to this therapy. One had a complete remission of injected and uninjected nodules and remains free of disease at 3 years following immunotherapy, and 2 others had dramatic regression of multiple metastatic nodules, with several nodules remaining dormant over a 2-year interval.

Using a highly precise technique developed in the Branch, serum alkaline ribonuclease levels have been determined in over 80 normal persons and age-correlated norms for ribonuclease reactivity have been derived. To date, over 90 cancer patients have had assay of serum ribonuclease levels prior to treatment. Markedly elevated levels of serum ribonuclease levels were found in patients with adenocarcinomas and squamous carcinomas. Paradoxically, patients with melanomas and sarcomas as a group have normal ribonuclease levels. There was no correlation between tumor stage and ribonuclease levels. It thus appears that this biochemical assay determines an early and tumor-specific change in patients with certain histologic types of malignancies. The use of this assay as an indicator of clinical cure in patients with adenocarcinomas and squamous carcinomas who have had definitive treatment of their malignancies is suggested.

The observation made by others that patients with lymphomatous tumors often have impaired in vitro lymphocyte reactivity to phytohemagglutinin (PHA) that correlates with the stage of disease has led to an investigation of the response of lymphocytes in autologous sera from patients with solid tumors to PHA. From the study of 120 normal subjects, age-correlated criteria of normal lymphocyte reactivity was established. Ninety-four patients have been followed for 3 years after surgical exploration, and it is now possible to correlate their preoperative lymphocyte reactivity with the subsequent clinical course. The lymphocyte reactivity of each patient was compared with the mean

age-correlated reactivity ±1 standard deviation (SD) of the controls. In each group of cancer patients there was a large percent, compared with controls, of individuals with lymphocyte reactivity less than 1SD of the mean reactivity of the controls. In the cured patient group, however, the percent with this low level of reactivity was smaller than in the inoperable or recurrent groups. Also in the cured patient group a larger percent, compared with the controls and the operable and recurrent tumor groups, had lymphocyte reactivity that exceeded 1SD of the mean reactivity of the controls. Patients with inoperable tumors and recurrent tumors have similar distribution of lymphocyte reactivity. All differences were statistically significant. The results showed that relatively high preoperative lymphocyte reactivity correlates with a favorable prognosis after definitive surgery for cancer, but relatively low lymphocyte reactivity, per se, does not indicate a poor prognosis. This study thus demonstrates that in vitro lymphocyte reactivity to PHA quantitates aspects of the host-tumor relationship that determine prognosis and suggests a use for the assay in determining the prognosis of patients who receive definitive surgical treatment of their malignancies. Lymphocyte reactivity is also determined in AB pooled sera to allow the separate assay of both the reactivity of lymphocytes of cancer patients and the effects of their sera on lymphocyte reactivity. Thus far, 103 patients with nonlymphoid tumors have been studied by this method. Cells and sera from 284 healthy volunteers provided age-correlated control data. Impaired lymphocyte reactivity and serum suppression of lymphocyte reactivity were demonstrated in patients with sarcomas and squamous carcinomas, but not in those with melanomas and adenocarcinomas as a group. The cellular and serum abnormalities coexisted in patients with sarcomas, but occurred independently in patients with squamous carcinomas. Similar incidences of abnormalities were present in patients with primary tumors only and those with metastases. These data indicate that this investigative system assays early and tumor-specific aspects of the host-tumor relationship and, therefore, are of potential usefulness in the study of factors associated with the induction of cancer and in monitoring the effects of tumor treatment.

Recently, a method of quantitating dinitrochlorobenzene (DNCB) contact sensitivity has been devised. With this technique, patients are scored with a quantitative scheme that distinguishes 4 levels of positive reactivity in addition to anergy. Using this quantitative method, sensitization to DNCB was studied in 143 healthy volunteers between the ages of 20-80 years. Among these subjects, 96.5% had a spontaneous flare reaction to DNCB. Thus far, over 150 cancer patients studied had a low incidence of spontaneous flare reaction (40.8% versus 96.5% in controls), a higher incidence of impaired reactivity (29.1% versus 0.7% in controls), and a higher incidence of anergy (30.1% versus 2.8% in controls). All anergic cancer patients developed a chemical irritation reaction to the sensitizing dose of DNCB. While abnormal reactivity occurred in all histologic tumor types studied, a significant relationship was shown between tumor histology and the distribution of abnormalities. Reactivity was most abnormal in patients with squamous carcinomas (43% anergic, 27% impaired reactivity), less in patients with melanomas (25% anergic, 44% impaired) and adenocarcinomas (26% anergic, 27% impaired), and least in patients with sarcomas (13% anergic, 25% impaired). With the exception of patients with sarcomas, the incidence of impaired cellular reactivity was similar in patients with clinically localized cancer (61%) and in patients with disseminated cancer (60%).

Studies with methylcholanthrene (MCA)-induced tumors demonstrate that the induction of host resistance to the growth of intramuscular (IM) tumor deposits of immunogenic tumors facilitate the survival and production of tumors when these cells are inoculated intravenously (IV). This phenomenon has been studied thus far with 5 MCA-induced tumors in C57Bl adult mice. Cells from 3 tumors, shown to be immunogenic by the failure of IM challenges of the tumor to grow after amputation of a growing tumor, produced similar incidences of tumors after IM and IV inoculation in nonimmunized mice. However, they caused more tumors after IV inoculation in immunized mice than after IV inoculation in nonimmunized mice or IM inoculation in immunized mice. Furthermore, decreasing numbers of IV inoculated tumor cells produced relatively more pulmonary tumors in immunized mice than in nonimmunized mice. By contrast, a nonimmunogenic MCA-induced tumor resulted in similar incidences of tumor after IV and IM inoculation in immunized mice; a weakly immunogenic and a nonimmunogenic tumor produced similar incidences of pulmonary tumors after IV inoculations in immunized and nonimmunized mice. A conclusion from this study is that cells from immunogenic MCA-induced tumors which enter the circulating blood are less susceptible to immune destruction than when they are in extravascular depots; conversely, host mechanisms of tumor destruction are less effective against these tumor cells when they are in the circulatory system than when they are in the extravascular sites. Because a possible explanation for these phenomena is the action of a factor in the circulating blood of immune animals which facilitates the survival and production of pulmonary metastases by IV inoculated tumor cells, and because of the accumulating evidence for tumor-protective factors in the circulating blood of cancer patients, it is important that the mechanism that gives rise to these phenomena be determined. In a study designed to further define the phenomenon of increased incidence of pulmonary metastases in immunized animals, mice inoculated with blood containing circulating tumor cells developed pulmonary metastases only when serum from the donor animals was inoculated with the cells. This suggests that the increased incidence of pulmonary metastases is due to serum factors produced in response to the tumor. Additional studies have determined the specificity of this phenomenon. In animals immunized with one immunogenic MCA-induced tumor, intravenously inoculated cells from the same and other antigenically distinct tumors all produced relatively more pulmonary embolic tumors in immune animals than in controls. These studies suggest that the tumor-facilitating factor may be nonspecific, at least in its action. Because of the relevance of this observation to the control of the dissemination of tumor in human cancer patients, further characterization of this factor is being actively pursued.

To test the hypothesis that antigenic differences exist between a primary tumor and metastases from it, the effect of immunization with a benzpyrene fibrosarcoma induced in syngeneic Fisher rats and metastases from the tumor have been studied in a system utilizing challenge with the primary and individual metastases. In these studies cells derived from transplanted metastases grew uninhibited in rats immunized with the primary, and further studies showed that the tumor metastases were immunogenically dissimilar not only from the primary but also from each other. These data offer considerable support of the concept that metastases may develop as a consequence of differential immunogenicity between the primary tumor and its metastases. Because of the obvious importance of these observations to the spread and control of tumor

metastases in humans and in the design of effective immunotherapy protocols utilizing tumor antigens, this experimental model is being adopted for the study of the differences in antigenicity between primary tumors and their metastases in human cancer patients.

The Tumor Immunology Section is investigating the properties of preparations rich in ribonucleic acid extracted from sites of antigen processing (such as the lymph nodes and spleen or peritoneal macrophages) of animals immunized with tumors in mediating immune responses to transplantation and tumor-specific antigens. This phenomenon has been investigated *in vivo* and *in vitro* utilizing heterologous as well as homologous and isologous sources of RNA. RNA was extracted from the lymph nodes and spleens of guinea pigs previously immunized with a chemical carcinogen induced sarcoma in C3H mice. Normal C3H spleen cells incubated with this RNA mixture and injected into C3H mice resulted in a marked decrease in the incidence of tumor growth when the C3H mice were subsequently challenged with live sarcoma cells. RNA from guinea pigs immunized with normal C3H tissues was ineffective. Treatment of the active RNA preparation with ribonuclease abolished activity while the deoxyribonucleic acid and pronase treatment did not. It has also been found that RNA from the nodes and spleens of guinea pigs immunized with a Fisher rat sarcoma induced by methylcholanthrene when mixed with dextran sulfate, a potent inhibitor of ribonuclease, and administered to normal Fisher rats showed a significant reduction in the incidence of tumor development upon live tumor cell challenge. *In vitro* experiments have also been performed. Normal strain 2 guinea pig spleen cells incubated with RNA extracted from the spleens of strain 2 guinea pigs immunized with methylcholanthrene-induced strain 2 sarcoma induced acellular areas of immune cytolysis in the guinea pig sarcoma cell monolayers. No cytolysis was produced when the RNA incubated spleen cells were added to normal strain 2 lung epithelial cell monolayers.

Cellular immune responses to tumor-specific transplantation antigens have been evaluated by a new assay for immune cytolysis using I^{125} iododeoxyuridine labeled target cells. This technique demonstrates the development of tumor-specific cellular immunity in animals. The I^{125} iododeoxyuridine label offers an advantage over other radioisotope labels in that it is not reutilized upon target cell death and leakage from live target cells is minimal. It has also been demonstrated that iododeoxyuridine labeled target cells are killed by cytotoxic antibodies, thus allowing this system to be used as an index of humoral cytotoxic antibodies. It has been reported that humans with skeletal and soft tissue sarcomas demonstrate lymphocyte cytotoxicity against sarcoma cells grown in tissue culture but not against normal skin cells from the same patient carrying the sarcoma. This demonstration of tumor-specific immunity by cell mediated lysis of tumor target cells could be blocked if serum from patients carrying progressively growing sarcomas was added to the target cells before the immune lymphocytes were added. Patients who were apparently cured of their sarcomas by surgical resection did not have serum which abrogated the lymphocyte mediated lysis of tumor target cells. A similar phenomenon has also been shown in strain 2 guinea pigs where serum from animals carrying progressively growing tumors abrogates the immune response of immune lymphocytes against target cells.

Studies using the isotope renogram in patients with ileal conduits have discovered that the sitting position is the preferred method used in this study.

If the renogram is performed in the customary supine position, a false obstructive pattern is most often obtained. The renogram has proven to be a valuable and important adjunct in the follow-up of patients following radical pelvic surgery and has been shown to be a sensitive indicator of obstruction. Since this obstructive pattern correlates well with the excretory urogram, a long-term follow-up of patients following pelvic surgery can best be accomplished with periodic renograms and flat plates of the abdomen rather than the customary excretory urogram.

Studies in both guinea pigs and mice have shown that subgrowth inoculum of tumor cells injected intradermally can prevent an intramuscular tumor challenge from growing. Such a means of immunoprophylaxis for immunotherapy is best shown in the guinea pig. Guinea pigs protected from an intramuscular tumor challenge by intradermal immunizations have been found to contain not only a cellular immune response, but a circulating humoral antibody which is specific for the immunizing tumor. This antibody appears to be of the IGM class and serum containing the antitumor antibody can successively transfer tumor-specific transplantation immunity.

Data obtained from mice regarding the role of the regional lymphatics to concomitant tumor immunity and that obtained from guinea pigs regarding the role of the regional lymphatics in both tumor allograft and isograft host recognition of tumor antigens have suggested that removal of the regional nodes would not adversely affect the host ability to mount a tumor-specific immune response.

Both a spontaneous murine renal adenocarcinoma and chemically-induced bladder tumors in mice have been shown to contain tumor-specific transplantation antigens. Chronic immunosuppression of mice ingesting a bladder carcinogen did not increase the incidence of bladder tumors, suggesting perhaps that the immune system has little effect on the initiation of chemically-induced bladder neoplasms.

Cryobiology: Efforts have been made to develop an animal model in which the effects of cryosurgery might be monitored biochemically. It has been demonstrated that cryodestruction of SV-40 induced prostatic tumors can be monitored by following acid phosphatase blood levels. Additional studies have been directed at potentiating the cryolesion. Specifically, inflow occlusion of hepatic blood flow was utilized while freezing hepatic tumors. It has been observed that repetitive freezes combined with inflow occlusion produce greater tissue destruction than a similar single freeze. These studies have allowed apparatus modifications to be developed to aid in standardizing cryodestruction and its measurement.

TABLE 1

Individual Written Intramural Consultations Received by Surgery Branch, NCI
Listed by Institutes
1963-1971

	NCI	NINDS	NIAMD	NHLI	NIAID	NIMH	NICHD	NIDR	NEI	EHS	TOTAL
1963	366	184	221	228	158	37	-	26	-	-	1220
1964	356	121	207	201	104	33	-	24	-	-	1046
1965	429	129	216	277	119	39	-	31	-	-	1240
1966	362	271	201	197	86	59	-	11	-	7	1205
1967	343	215	245	166	85	61	-	17	-	-	1132
1968	278	143	192	203	111	54	-	25	-	7	1013
1969	314	131	248	207	128	45	-	31	-	10	1114
1970	199	59	137	104	96	41	-	12	-	7	655
1971	220	98	152	89	94	62	15	28	28	18	804

TABLE 2

Summary of Written Extramural Consultations Requested by or Through
Surgery Branch, NCI
1963-1971

	ENT	General Surgery	Gynecology	Urology	Oncology	Total
1963	346	14	7	18	9	394
1964	233	6	4	4	3	242
1965	268	6	2	9	1	286
1966	271	8	4	13	2	298
1967	294	11	1	1	1	308
1968	306	8	1	1	1	317
1969	329	8	2	3	2	344
1970	778	12	2	2	0	794
1971	704*	3	0	0	0	707

*56% of these consultations were at the request of Dr. Robert I. Henkin, NHLI,ET, part of his clinical research program.

TABLE 3

Annual Report of NIH Surgical Suites
Listing the Number of Major & Minor Procedures
Carried Out by Each of the Operative Units at NIH
April 1, 1971 - March 31, 1972

Patient		NCI	NHLI	NINDS Neuro.	NINDS Muscle	NEI	NICHD	NIDR	Cons.	Total #Pts. Operated	
NCI	Major	197	2	7	0	4	5	0	4	219	553
	Minor	304	1	1	12	2	3	4	7	334	
NHLI	Major	15	236	0	0	3	1	1	1	257	341
	Minor	30	32	7	2	1	7	2	3	84	
NINDS	Major	5	1	83	0	1	0	0	1	91	347
	Minor	8	1	11	231	0	1	1	3	256	
NIAMD	Major	37	0	0	0	0	1	0	7	45	75
	Minor	17	0	0	6	1	0	1	5	30	
NIAID	Major	5	0	0	0	0	0	0	6	11	55
	Minor	33	0	1	6	0	1	0	3	44	
NIDR	Major	0	0	0	0	0	0	3	0	3	3
	Minor	0	0	0	0	0	0	0	0	0	
NIMH	Major	1	0	0	0	0	0	0	1	2	7
	Minor	3	0	1	0	0	1	0	0	5	
NICHD	Major	5	0	1	0	0	5	0	2	13	70
	Minor	20	0	0	0	3	34	0	0	57	
NEI	Major	0	0	0	0	44	0	0	0	44	75
	Minor	0	0	0	0	30	0	0	1	31	
GRAND TOTAL		680	273	112	257	89	59	12	44		1526

TABLE 4

Number of Surgical Procedures Performed
by NCI Surgery Branch Staff
and Magnitude of the Procedures in 1971

	10 East	Other NCI	Other Institutes	Total
1963	470	159	399	1028
1964	424	215	256	895
1965	394	112	199	705
1966	410	113	218	741
1967	440	145	140	725
1968	394	205	113	712
1969	339	233	158	730
1970	301	273	230	804
1971	228	273	179	680
Major	125	72	87	284
Minor	103	201	92	396

Serial No. NCI-3800
1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Removal of Cancer and Other Tissues for Histologic Biochemical and Other Studies as Required by Scientists and Other Investigators for Correlated Studies

Previous Serial Number: Same

Principal Investigators: Entire Staff of the Surgery Branch, NCI

Other Investigators: Several Branches of the NCI and many members of other Institutes with whom cooperative studies develop, particularly G.D. Aurbach, M.D., P.P. Carbone, M.D., V.T. DeVita, M.D., E.S. Henderson, M.D., K. Engelman, M.D., R.I. Henkin, M.D., R.E. Johnson, M.D., N. Talal, M.D., A.D. Steinberg, M.D., D.H. Van Thiel, M.D., R.J. Sherins, M.D., F.C. Bartter, M.D., L.B. Thomas, M.D., R.A. Malmgren, M.D., J. Zajicek, M.D., and J. Linsk, M.D.

Cooperating Units: Reproductive Study Branch, NICHD; NIAMD; Anesthesiology Department, Clinical Center, and all clinical units of the NIH, Cytophatology Department, Karolinska Institute, Stockholm, Sweden; and Cytopathology Department, Atlantic City Hospital, Atlantic City, New Jersey

Man Years:

Total:	6-1/2
Professional:	4
Other:	2-1/2

Project Description:

Part I. Service Aspects

Part II. Specific Cooperative Studies

A. Pheochromocytoma

B. Hyperparathyroidism

C. Thyroid Carcinoma

D. Lymphoma and Hodgkin's Disease

E. Mechanism of Taste and Smell

F. Splenectomy and its Role in Chronic Myelogenous Leukemia

G. Fine Needle Aspiration Biopsy in the Diagnosis of Solid Tumors with Cytologic Techniques

H. Therapy of Systemic Lupus Nephritis, a Controlled Study Comparing Cyclophosphamide, Azathioprine, and Placebo

I. Testicular Disfunction Following Cyclophosphamide Therapy for Hodgkin's Disease

Project Description:

Part I. Service Aspects

Objectives:

The object of this project is to make available human tissue for histologic, histochemical, biochemical, biophysical, viral, and immunological studies to scientists working in various Branches of the National Cancer Institute and other Institutes who require tissue for studies. As a rule, such studies are not done as cooperative projects. In addition, it implies furnishing general surgical consultative coverage of patients admitted to other Branches of the National Cancer Institute and/or other Institutes and assisting with their study projects. This surgery is often definitive in nature, the therapy regime requiring surgical tissue excision as the sole type of therapy or more often as an adjuvant to other modes of treatment.

Methods Employed:

Methods employed are standard surgical procedures. Material from this project is obtained mostly from patients hospitalized for study in other Branches or Institutes. Occasionally it is necessary to admit patients for specific studies in which a specific type of tissue is obtained. Tumor tissue, lymph nodes, muscle, skin, and liver and spleen comprise the greater share of tissue removed.

Major Findings:

In that the major portion of the work involved in this project is done as a surgical consultative service and not as a cooperative study, the project results are not immediately available to the surgical service. During the course of supplying this service, 804 individual requests for consultation were received. These figures do not include the extensive follow-up which is afforded each of these consultations but only the original written request to participate in their care. Of these requests 452 resulted in an operative procedure, 35% of which entailed major surgery. See attached Tables 1-4.

Significance to Biomedical Research and Program of the Institute:

This project is a service function for other Branches of the National Cancer Institute and National Institutes of Health. It is seldom that the Surgery Branch is included in the research studies other than supplying the tissue to the investigator; however, this trend is being reversed and several studies are being carried out on a cooperative basis with Surgery Branch staff now serving as active counselors in the overall study.

Proposed Course:

Continued cooperative assistance within the physical limits of the Surgery Branch staff.

Project Description:

Part II. Specific Cooperative Studies

A. Pheochromocytoma

Objectives:

The goals of this project have been: 1) the development and evaluation of a joint medicosurgical program for the overall management of patients with pheochromocytoma and 2) continuing clinicopathologic scrutiny of the operative material from these cases in an effort to define more clearly the malignant potential possessed by this unusual tumor which lacks the usual histologic criteria for malignancy in spite of demonstrating its biologically malignant nature in about 10% of cases.

Methods Employed:

Standard surgical and anesthetic procedures are used when operating upon these patients. The analytical methods for catecholamine determinations are those developed and currently employed by the Experimental Therapeutics Branch, NHLI. The patients studied are those who have been referred to the Experimental Therapeutics Branch, NHLI, for evaluation and treatment of their pheochromocytoma. Both conventional light and electron microscopy are applied to the study of the resected materials. Attempts are underway to grow these tumors in tissue culture in order to develop a means for assessing biologic malignant potential by an in vitro procedure.

Major Findings:

Our experience now includes 29 cases and previous conclusions with respect to management of these patients have been reaffirmed. Intraoperative localization of tumor by observation of blood pressure rise in response to manipulation remains satisfactory. Thorough preoperative biochemical preparation of these patients continues to provide a much safer anesthetic and operative

course. More data has accumulated to strengthen our concept that total adrenalectomy should be done in all instances of adrenal pheochromocytoma rather than attempt to shell tumor out of a gland which is to be left behind. Preliminary results with tissue culture of pheochromocytoma material suggest that a clinical and histologically benign pheochromocytoma currently cannot be developed into a serially transferable tissue culture line. Further experience with a more malignant lesion is necessary.

Significance to Biomedical Research and Program of the Institute:

There are few tumors which have a dual uniqueness similar to pheochromocytoma, i.e., a functional lesion with an easily measured secretory produce but which lacks intrinsic histologic criteria of malignancy. This combination provides a rare opportunity to study the effectiveness of vigorous surgical therapy using as an endpoint something other than the ultimate demise of the patient from disseminated disease.

The demonstration that these tumors will grow for a time in tissue culture, although their transferability into a permanent line has not been achieved as yet, suggests that this means may be useful in assaying degree of malignant potential prior to the development of lethal biological evidence in the patient. The measurement of catecholamines in the tissue culture supernatant and in the cells themselves offers further potential of evaluating efficacy of agents that might have specific antitumor activity.

Proposed Course:

The cooperative study has now concluded and the review that is underway will have emphasis placed upon elucidation of relative degrees of malignancy among the various lesions and upon attempts to benefit more patients with malignant pheochromocytomas by meticulous surgery.

Project Description:

Part II. Specific Cooperative Studies

B. Hyperparathyroidism

Objectives:

The Clinical Endocrinology Branch, NHLI, the Section on Mineral Metabolism, Metabolic Diseases Branch, NIAMD, and the Diagnostic Radiology Branch, Clinical Center, have programs underway to determine clinical, laboratory, and radiographic criteria which justify surgical exploration of the neck for the treatment of parathyroid disease. This joint program is expected to increase the reliability of diagnosis and efficacy of treatment for parathyroid disorders, particularly in early cases where the treatment will reduce the incidence of secondary renal disease.

Methods Employed:

Standard surgical and anesthetic procedures are used for operating on the patients. In addition to the customary extensive clinical evaluation and laboratory determination of static derangements of calcium and phosphorus metabolism, a battery of tests are performed to measure responses to induced distortions of calcium and phosphorus hemostasis. Additionally, selective inferior thyroid arteriographic studies are done preoperatively, as is selective venography and removal of blood samples from various veins draining the parathyroid complex identified by venography. Radioimmunoassay determinations of parathyroid hormone are made upon the venous samples so obtained. At operation, prior to any extirpative procedure, intensive efforts are made to identify positively by frozen section all parathyroids present.

Major Findings:

Selective inferior thyroid arteriography continues to be of considerable value in the preoperative localization of abnormal parathyroid glands, particularly when the subtraction technique is applied to the arteriograms. Selective venography has not been helpful in finding abnormal parathyroid glands, but has enabled the radiographer to identify the appropriate veins from which to withdraw blood for parathyroid hormone assays. The parathyroid hormone assays have been extremely encouraging in that out of 20 patients studied the lateralization of the parathyroid neoplasm has been successfully predicted preoperatively in all 20 cases.

Long-term follow-up studies 8 to 11 years after exploration and removal of abnormal parathyroid tissue from chronic renal stone formers with normocalcemic hyperparathyroidism have provided support for the concept that early operation is advantageous for decreasing stone formation and preventing subsequent loss of functional renal mass.

Significance to Biomedical Research and Program of the Institute:

Early diagnosis and removal of overactive parathyroid tissue is an important step toward preservation of renal function from the ravages of stone formation. The addition of selective venous catheterization and radioimmunoassay for parathyroid hormonal levels of samples thereby obtained is an important tool for both diagnosis of primary hyperparathyroidism and preoperative localization of the abnormal tissue. Selected angiography has continued to bear out its earlier promise as an important aid to the surgeon in finding the abnormal glands readily, with a resultant decrease in operating time and morbidity.

Proposed Course:

Continued cooperative studies.

Project Description:

Part II. Specific Cooperative Studies

C. Thyroid Carcinoma

Objectives:

To assess the clinical, pathological, and endocrinological parameters of patients with Sipple's syndrome (medullary carcinoma of the thyroid gland, pheochromocytoma, and parathyroid hyperplasia).

Methods Employed:

Presently a large number of patients have been admitted to the NIH with the diagnosis of Sipple's syndrome. These patients have been diagnosed positively by the presence of an elevated thyrocalcitonin level in their peripheral blood. Patients with peripheral levels above 0.3 nanograms/ml. can be diagnosed with 100% accuracy as having medullary carcinoma of the thyroid gland. A large number of these patients have concurrent pheochromocytomas and parathyroid hyperplasia. Large numbers of this family have been screened and admitted to the NIH for study. The disease status is diagnosed and they subsequently undergo operation for removal of the thyroid carcinoma and other diseased organs should they be manifest.

Major Findings:

The elevated peripheral level of thyrocalcitonin has been an excellent screening test for the presence of medullary carcinoma of the thyroid gland. We have had no false positive findings in that all patients with levels above 0.3 nanograms/ml. have thyroid carcinoma. At present, of all of the serological tests for the detection of cancer, this one has the greatest accuracy. It has also been found that histaminase levels are elevated once the disease metastasizes outside of the thyroid gland. This is a good index of measuring extent of disease in those patients who either have undergone surgery or in those who have recurred or who have residual tumor left behind following surgery. The standard operative procedure that has been employed in these patients has been a total thyroidectomy with removal of the central nodes of the neck. Most of the patients in our group have had generalized parathyroid hyperplasia and have undergone subtotal parathyroidectomy.

Significance to Biomedical Research and Program of the Institute:

This methodology has been extremely important in that the serological screening test has had a high degree of accuracy in the thyroid carcinoma. This allows the early diagnosis of medullary carcinoma of the thyroid gland and hopefully total removal of the neoplasm before metastasis has occurred.

Proposed Course:

To screen the remainder of the pedigree and admit those diseased for study and surgery.

Project Description:

Part II. Specific Cooperative Studies

D. Lymphoma and Hodgkin's Disease

Objectives:

To assess the adequacy of staging of patients with lymphoma and Hodgkin's disease by nonsurgical methods. To assess the effect of splenectomy on patient survival and tolerance of radiotherapy and chemotherapy treatment regimens.

Methods Employed:

All patients admitted to the Chemotherapy and Radiotherapy Services with lymphoma and Hodgkin's disease undergo routine staging by the combined evaluation of clinical and physical findings, liver chemistries, lymphangiograms, and chest x-ray. To assess the accuracy of these staging techniques, selected patients have been subjected to laparotomy and biopsy of the liver and abdominal lymph nodes. Selected adenopathy detected after treatment is further evaluated by biopsy and histologic examination.

Major Findings:

The mounting experience gained through the examination of tissues removed at laparotomy in patients with lymphoma and Hodgkin's disease in comparison with staging data will more accurately delineate those patients who will profit from the inconvenience of laparotomy. Sufficient evidence has accumulated to allow the conclusion that all patients will not benefit from staging laparotomy, a conclusion that is at variance with the findings of several other major treatment centers. On the other hand, biopsy of multiple abdominal nodal areas has demonstrated the limitations of lymphangiography in assessing the extent of abdominal nodal disease. The contribution made by abdominal laparotomy and biopsy, however, is clearly dependent on the treatment proposed and how it is altered by the finding of unsuspected positive abdominal lymph nodes.

The evidence emerging from other treatment centers that splenectomy favorably affects patient survival and tolerance of radiotherapy and chemotherapy in Hodgkin's disease and lymphosarcoma is currently being assessed at this institution by a prospective study of the effects of splenectomy on these parameters. Although the value of splenectomy in those patients with abnormal consumption of blood cells is continuously being demonstrated as is the laboratory benefit of splenectomy and the salutory benefit of removal of spleens unresponsive to

chemotherapy and radiotherapy, sufficient data has not yet accumulated to allow assessment of the effects of splenectomy on tolerance of subsequent specific treatment and overall survival.

Significance to Biomedical Research and Program of the Institute:

The cooperation of the Surgery Branch with the divisions of the Institute concerned with treatment of patients with lymphoma and Hodgkin's disease results in a continuously improving program of diagnosis and treatment of these patients. These studies allow the intelligent selection of those patients who would benefit by laparotomy prior to specific treatment of the disorder.

Proposed Course:

Studies in progress will correlate laparotomy findings in these diseases with response to therapy and long-term prognosis. The effect of splenectomy on tolerance to chemotherapy and radiotherapy will be determined.

Project Description:

Part II. Specific Cooperative Studies

E. Mechanism of Taste and Smell

Objectives:

To investigate the mechanism of taste and smell and the compensatory alteration in these functions following radical head and neck surgery.

Methods Employed:

Subjective reports of olfactory acuity were obtained in 35 patients who had undergone laryngectomy for carcinoma of the larynx and 17 patients who had undergone paranasal sinus exenteration. Each was questioned about his ability to smell before and after surgical removal of the larynx. Detection and recognition thresholds for the vapors of pyridine, thiophene, nitrobenzene, amyl acetate, and camphor were determined objectively in 18 of these patients. Eight were studied both before and after laryngectomy, the others only after operation. Olfactory thresholds were determined by a slight modification of a forced choice-three stimulus sniff technique.

A detection threshold was defined as the lowest concentration of solute and solvent that the patient could consistently discriminate from two solutions of solvent alone. A recognition threshold was defined as the lowest concentration of solute and solvent that the patient could consistently identify correctly as the solute. All thresholds were determined in the afternoon in a room from which extraneous odors had been excluded. The patients had not eaten or smoked for at least an hour before the examination. Subjective reports of olfactory acuity and olfactory thresholds were obtained in some

patients as soon after operation as they were alert enough to make meaningful judgments; in some patients this was within 2 hours after operation.

Detection and recognition thresholds for representatives of each of the 4 taste modalities were also obtained in each of the patients in whom these measurements could be obtained.

Major Findings:

We have found in 25 patients after removal of the olfactory nerve and primary olfactory epithelium that these patients were still able to detect odors using accessory areas of olfaction of the oropharynx. In 35 patients after total laryngectomy in which the primary olfactory area was not surgically disturbed all patients lost their ability to detect odors in a normal fashion.

In both groups of patients after surgery there was loss of the normal ability to detect and recognize vapors through the olfactory nerve-olfactory epithelium relationship. Only in the patients having sinus exenteration was this inter-relationship disturbed surgically. In the patients having laryngectomy loss of olfactory acuity, this appeared to be unrelated to surgical trauma to the first cranial nerve or to significant changes in air flow. However, in both groups, shortly after surgery, patients were able to detect and in some cases recognize odors through the use of accessory areas of olfaction located in mucosal surfaces on the lateral walls of the nares, oral cavity, and pharynx, these areas being innervated by the fifth, ninth, and tenth cranial nerves. Although the olfactory acuity obtained through use of the accessory areas was markedly depressed compared with that of normal subjects, it could be improved with the oropharyngeal inhalation of vapors. A stable level of olfactory acuity was usually reached by 18 months after surgery where it remained for as long as 8 years after operation.

Significance to Biomedical Research and Program of the Institute:

The mechanism responsible for the hyposmia following laryngectomy is not clear. It is not related to surgical interference with the first cranial nerve, since surgery did not involve any anatomical disturbance of that area. It is unlikely that the hyposmia is related to a physiological alteration in the olfactory epithelium, since it was noted subjectively and measured.

Proposed Course:

A continued cooperative effort is planned, studying selected patients who have any physical alteration of their head and neck anatomy, in order to better identify the true mechanisms of taste and smell.

Project Description:

Part II. Specific Cooperative Studies

F. Splenectomy and its Role in Chronic Myelogenous Leukemia

Objectives:

To reassess the effect of splenectomy on normalization of blood cell kinetics in patients with chronic myelogenous leukemia (CML); to delineate those patients that will benefit from splenectomy.

Methods Employed:

Thrombocytopenia is usually a late manifestation of CML. Most often it is associated with transformation of the disease to the blastic phase. In a smaller number of patients thrombocytopenia, which may be a direct manifestation of the disease process or result from an inordinate sensitivity to the myelosuppressive effects of chemotherapy, occurs in the chronic stage. This latter instance severely limits the effective treatment of the disease and may require hospitalization and platelet transfusion support. In 6 patients presenting with this clinical picture splenectomy was performed. The effects of this operative procedure on the subsequent hematologic picture and tolerance to therapy were evaluated.

Major Findings:

The indications for splenectomy in this group of patients fell into two general categories: (1) The inability to control the clinical and hematological manifestations of the disease because of an inordinate sensitivity of platelet blood counts to busulphan; and (2) prolonged pancytopenia and severe thrombocytopenia secondary to busulphan myelosuppression, resulting in frequent hospitalization for multiple platelet transfusions. All patients tolerated the operation without excessive bleeding. Platelet concentrate transfusions were utilized throughout the procedure and during the postoperative period. There was an almost immediate rise in the platelet count within 48 hours in all patients. The extent of postoperative thrombocytosis did not appear to correlate with spleen size at the time of surgery. In one case the spleen histology predicted blastic transformation, showing extensive replacement of the spleen by immature blast cells. In the other 5 cases, the histopathology was compatible with CML or myeloid metaplasia. There appeared to be no correlation between the numbers of megakaryocytes in the marrow and subsequent response to therapy. The postoperative rise in platelet count was rapid in 3 patients and less so in 2 patients. In all 5 patients with hemorrhagic manifestations of thrombocytopenia, splenectomy ameliorated the bleeding and obviated the need for further platelet transfusion.

Significance to Biomedical Research and Program of the Institute:

This study delineates a role for splenectomy for the treatment of CML. Those patients with prolonged thrombocytopenia and hemorrhagic manifestations due to busulphan-related myelosuppression unresponsive to corticosteroids and frequent platelet transfusions and those with inordinate platelet sensitivity to chemotherapy are candidates for splenectomy. The rapid rise in platelet count and cessation of hemorrhagic complications were gratifying results in these patients. This information is of critical importance to all involved in the exploration of improved therapeutic approaches to the treatment of this disease.

Proposed Course:

This project will continue, with further exploration of the beneficial effect of splenectomy on the course of patients with CML. These include exploration of less obvious effects, including immunologic alterations, precipitated by splenectomy.

Project Description:

Part II. Specific Cooperative Studies

G. Fine needle Aspiration Biopsy in the Diagnosis of Solid Tumors with Cytologic Techniques

Objectives:

For the past 15 years at the Karolinska Institute in Stockholm, Sweden, fine needle aspiration biopsy techniques have been employed successfully in the diagnosis of tumors in the breast, salivary glands, thyroid, lymph nodes, prostate, pelvis, and lung. The incidence of false positives has been zero and false negatives approximately 10%. A fine 22-gauge needle is employed, anesthesia is unnecessary and patients do not object to the procedure. The technique has not gained favor in this country in the diagnosis of solid tumors because of the manner in which the slides have had to be prepared and the technique carried out as described in publications from Stockholm. That is: (1) A cytologist does the aspiration; (2) the 1 or 2 drops of material contained in the needle following aspiration is carefully smeared on a slide and air dried; (3) the slide is then stained with a hematology stain for interpretation. This intermixing of clinician, hematologist, and cytologist in one individual, while it has worked well at the Karolinska Institute, has not been able to work well in this country.

Our objective is to evaluate the technique of fine needle aspiration used in Stockholm by employing both the Stockholm method of preparing and interpreting the smears and conventional cytological methods of preparation and interpretation as is used in this country by most cytologists. It is felt that if it can be demonstrated that the method is of value and that interpreation is satisfactory using the Papanicolaou technique that the procedure will gain

favor in this country and lead to earlier diagnosis of solid tumors and in addition assist in the management of metastatic disease.

Methods Employed:

Undiagnosed solid tumors will be aspirated using the fine needle (gauge 22) technique as used in Stockholm. Two aspirations will be made of each tumor aspirated and slides prepared air dried and stained with the hematology stain and, in addition, slides will be prepared for the Papanicolaou stain. The syringe and needle will be washed with saline and this then put through a millipore filter and stained with the Papanicolaou stain. The Papanicolaou slides will be read by cytologists at NIH, the hematology stain slides will be read by Dr. Linsk and Dr. Zajicek.

Whenever possible tumor masses which have been aspirated and then later excised will have touch prints prepared of the freshly cut tumor surface and stained with both the Papanicolaou stain and a hematology stain. All slides will be kept on file here at NIH, so that depending on the most effective method of staining arrived at, these slides can then be used to train other cytologists at the Institution and across the country in the interpretation of aspiration biopsy smears. It may turn out that while one stain is more accurate in diagnosing tumors in one location another stain is more useful for tumors in another area.

Major Findings:

Thus far aspiration biopsy has been performed on 150 solid tumors. There has been good cytological and pathological correlation with no false positives. The accuracy has been 95% in diagnosing tumor in lymph nodes and 93% in diagnosing tumor in subcutaneous nodules. Of 32 breast aspirations performed for breast tumor only fluid was obtained in 6 making excisional biopsy unnecessary. In 9 aspirations the histological diagnosis was cancer and in 6 of these the cytological interpretation of the aspirate was cancer.

In the 3 aspirations called negative cytologically the needle did not enter the tumor in 1 and in 2 only blood was obtained. It is anticipated that with further development of the technique the incidence of false negative reports should be 5% or less and that the patient with a breast mass will be spared excisional biopsy 70% of the time. We have found the simultaneous preparation of both Pap smears and smears stained with Giemsa to be most effective for proper interpretation.

Besides cytological diagnosis of tumors with this technique we have been successful in growing in tissue culture sarcoma, adenocarcinoma, melanoma, and breast cancer from cells aspirated with a #22 needle. The method also has value in the cytological grading of tumors.

Significance to Biomedical Research and Program of the Institute:

We are constantly in search of a method of earlier diagnosis of tumors in all parts of the body. This aspiration biopsy technique utilizing a fine needle provides a means of giving quick, accurate diagnosis of cancer if it is present in 90% of the tumor masses aspirated. If with this technique a mass as small as 0.5 cm. may be aspirated and diagnosed, and the diagnosis of cancer can be rendered with a fine needle aspirate instead of incisional or excisional biopsy, treatment of the patient will be considerably accelerated and the cost significantly reduced. The procedure in the Stockholm series has proven to be of most value in evaluating patients with breast cancer, prostate cancer, or salivary gland cancer. It is a technique which can be utilized readily in any screening clinic and should significantly enhance the earlier diagnosis of cancer if it is present.

Proposed Course:

Because of its importance in the overall cure of cancer through earlier diagnosis this project is being given the wholehearted support of all participants. It will take precedence over all other studies in the coming year.

Project Description:

Part II. Specific Cooperative Studies

H. Therapy of Systemic Lupus Nephritis, a Controlled Study Comparing Cyclophosphamide, Azathioprine, and Placebo

Objectives:

Although steroids adequately control the extrarenal manifestations of SLE, they are often ineffective in preventing the progressive deterioration of renal function. Despite short-term control of nephritis, renal function gradually deteriorates and corticosteroids are gradually increased until the undesirable side effects of steroids are as life threatening as the renal disease itself. Cytostatic therapy has been used in recent years in an attempt to reduce the dose or to obviate the need for steroids. Azathioprine has been most widely used in SLE renal disease with reported encouraging results. Cyclophosphamide has also been shown to reduce serum concentration of anti-DNA and anti-RNA antibodies and in some patients improves renal function. However, cyclophosphamide is a highly toxic agent when administered in continuous daily doses. The purpose of this study is to compare cyclophosphamide and azathioprine in the therapy of SLE nephritis and to compare the efficacy of the two drugs. A secondary objective is to continue to obtain information bearing on the relationship of various laboratory parameters to SLE renal disease activity.

Methods Employed:

All patients admitted to this study must have: (1) A diagnosis of SLE based on the ARA criteria. (2) A positive SLE cell preparation sometime in the course of the disease. (3) The presence of active kidney disease unaccounted for by other pathologic processes. Active renal involvement will be based upon one of the following criteria, after several serial determinations over a 2 week period: (a) The presence of RBC casts in a fresh spun urine sediment; (b) hematuria greater than 10 RBC/HPF or pyuria greater than 10 WBC/HPF plus granular casts; (c) Proteinuria greater than 1 gm/24 hours and low serum complement; (d) histologic evidence of active nephritis plus either low serum complement or the presence of serum anti-DNA antibodies. (4) The informed consent of the patient to all aspects of the study. Patients excluded from this study will be the following: (1) A major infection within 2 weeks of onset of therapy. (2) Pregnancy or its anticipation during the study. (3) A total white count of less than 2,000. (4) History of sensitivity to the study drugs or related drugs. (5) Cytostatic therapy within 8 weeks. (6) Platelet count of less than 100,000. After the evaluation period, the patients will be admitted to the study randomly and assigned to one of the three treatment groups (a) cyclophosphamide, (b) azathioprine, (c) placebo. The drug or placebo will be administered in a double blind fashion in the hospital for 10 weeks. At the end of this period each patient will be individually evaluated as to the efficacy of the treatment they received and an appropriate long-term treatment program set up under supervision of the NIH follow-up clinic when possible. The study plan will be altered if the patient should have an onset of precipitous renal failure or a life threatening infection should ensue or serious cyclophosphamide or azathioprine toxicity should be incurred.

Major Findings:

Although the study is not completed, the initial findings suggest that both azathioprine and cyclophosphamide are effective in treating the renal disease associated with SLE. There is a suggestion, however, that perhaps these two drugs may act differently.

Significance to Biomedical Research and Program of the Institute:

Both animal studies and early clinical studies suggest that cytostatic agents may have a therapeutic role in the renal disease associated with lupus erythematosus. It is hoped that this study will resolve many of the uncertainties concerning cytostatic drugs which currently exist in the treatment of SLE. A controlled study such as the one underway seems to be necessary to answer these questions.

Proposed Course:

To continue the present study, and perhaps initiate a new study in the future which may include a combination of drug therapy.

Project Description:

Part II. Specific Cooperative Studies

I. Testicular Disfunction Following Cyclophosphamide Therapy for Hodgkin's Disease

Objectives:

Increasing numbers of young adults with Hodgkin's disease are receiving x-ray therapy and/or chemotherapy and achieving a clinical remission or cure. Remissions are now of sufficient length that post-treatment morbidity from primary chemotherapy becomes apparent, and will become increasingly important as success with chemotherapy improves. Azospermia is a prominent feature of those men treated with alkylating agents and is a major concern to young men who survive. We propose to determine whether replacement of gonadotropins are indicated for restoring the viable germinal epithelium. Men following chemotherapy are clinically well androgenized. There is little evidence to suggest a defect in pituitary Leydig cell function. However, the azospermia defines damage to the germinal epithelium. Though not specifically known, there is a feedback relationship between the germinal epithelium and FSH release. We propose to study this feedback and determine if: (1) FSH is elevated, (2) the anatomic level of the feedback is in the testes, and (3) the alkylated agents are somehow interfering with feedback to the pituitary. In terms of therapy, the justification for gonadotropin replacement will depend on evidence of inadequate FSH secretion. Our experience with the biologic effects of irradiation on the human testes show that denuding of the germinal epithelium and subsequent readmission of the spermatogenesis are dose-related. We propose to correlate the dose of alkylating agents with extent and duration of damage to the germinal epithelium.

Methods Employed:

1. Evaluation of the germinal epithelium-FSH axis.
   a. Nine separate semen specimens will be obtained after 2 or 3 days sexual abstinence and be examined for sperm morphology and motility.
   b. A testicular biopsy will be obtained under local anesthesia while the patient is hospitalized. The testicular tissue will be examined using H and E Masson trichrome and Weigert stains to evaluate morphologic appearance and gametogenesis.
   c. Twenty-four hour urine will be obtained and FSH content measured using the Steelman-Pohley assay. Three 10 cc specimens of blood will be obtained on different days for plasma FSH as determined by radioimmunoassay methods.
2. Evaluation of the Leydig cell-LH axis.
   a. Three 24 hour urines will be obtained for total gonadotropins.
   b. Three blood samples will be obtained for plasma LH and testosterone.
3. Evaluation of gonadotropin secretion.
   Clomiphene citrate 200 mg/day will be given orally for 5 days. Blood

will be drawn for plasma FSH, LH and testosterone on days 0, 5, and 7. A semen specimen will be obtained and examined on the 7th treatment day.

4. Therapy.
   a. Pergonal will be given I.M. daily for 4 to 16 weeks if any evidence of decreased FSH secretion is found.
   b. Semen specimens will be obtained weekly during the Pergonal therapy.
   c. A repeat testicular biopsy will be obtained when the semen is normal to examination or at the end of the Pergonal therapy period.
   d. The patient will be followed as an outpatient in the clinic.

Major Findings:

We have studied 7 men who have been in remission from their Hodgkin's disease for 1-5 years after treatment to evaluate the interaction of the testicular components and the gonadotropin secretion. Persistent azospermia was present in 6/7 men. Testicular biopsy of these azospermic men showed complete absence of all germinal elements; however, Sertoli cells and Leydig cells were present. Plasma FSH was elevated 5-fold in each man compared to normal men. One patient with a normal sperm count 2 years after chemotherapy had complete spermatogenesis on biopsy and normal plasma FSH. In contrast, serum LH was normal in all men as plasma testosterone was normal in all but one. Furthermore, Clomiphene 200 mg/day for 5 days produced increases in serum FSH, LH and plasma testosterone, demonstrating the appropriate pituitary-Leydig cell responses. An additional azospermic patient with lymphoma had received a full course of chemotherapy 2 months before study, but was not in remission. Plasma FSH was normal despite the presence of only spermatogonia and Sertoli cells in the testicular biopsy suggesting that later stages of spermatogenesis do not affect FSH secretion. As with other men, serum LH, T, and responses to Clomiphene were normal.

Significance to Biomedical Research and Program of the Institute:

Since FSH levels are lower in these patients than in the castrated men, we conclude that all FSH may be modulated in part by Leydig cells and/or testosterone, other factors related specifically to the germinal epithelium are important. We suggest that the source of the testicular factor involved in FSH feedback control is either the Sertoli cell or the early germinal elements.

Proposed Course:

This retrospective study will be continued along with a prospective study. In addition, a sperm bank is being organized prior to therapy for Hodgkin's disease and methods will be employed to try to prevent the effects of alkylating agents on the germinal epithelium of the testes.

TABLE 1
Individual Written Intramural Consultations Received by Surgery Branch, NCI
Listed by Institutes
1963-1971

	NCI	NINDS	NIAMD	NHLI	NIAID	NIMH	NICHD	NIDR	NEI	EHS	Total
1963	366	184	221	228	158	37	-	26	-	-	1220
1964	356	121	207	201	104	33	-	24	-	-	1046
1965	429	129	216	277	119	39	-	31	-	-	1240
1966	362	271	201	197	86	59	-	11	-	7	1205
1967	343	215	245	166	85	61	-	17	-	-	1132
1968	278	143	192	203	111	54	-	25	-	7	1013
1969	314	131	248	207	128	45	-	31	-	10	1114
1970	199	59	137	104	96	41	-	12	-	7	655
1971	220	98	152	89	94	62	15	28	28	18	804

TABLE 2
Summary of Written Extramural Consultations Requested by or Through
Surgery Branch, NCI
1963-1971

	ENT	General Surgery	Gynecology	Urology	Oncology	Total
1963	346	14	7	18	9	394
1964	233	6	4	4	3	242
1965	268	6	2	9	1	286
1966	271	8	4	13	2	298
1967	294	11	1	1	1	308
1968	306	8	1	1	1	317
1969	329	8	2	3	2	344
1970	778	12	2	2	-	794
1971	704*	3	-	-	-	707

*56% of these consultations were at the request of Dr. Robert I. Henkin, NHLI,ET, part of his clinical research program.

TABLE 3

Annual Report of NIH Surgical Suites
Listing the Number of Major & Minor Procedures
Carried Out by Each of the Operative Units at NIH
April 1, 1971 - March 31, 1972

Patient		NCI	NHLI	NINDS Neuro.	NINDS Muscle	NEI	NICHD	NIDR	Cons.	Total Pts. Operated	#Pts.
NCI	Major	197	2	7	0	4	5	0	4	219	553
	Minor	304	1	1	12	2	3	4	7	334	
NHLI	Major	15	236	0	0	3	1	1	1	257	341
	Minor	30	32	7	2	1	7	2	3	84	
NINDS	Major	5	1	83	0	1	0	0	1	91	347
	Minor	8	1	11	231	0	1	1	3	256	
NIAMD	Major	37	0	0	0	0	1	0	7	45	75
	Minor	17	0	0	6	1	0	1	5	30	
NIAID	Major	5	0	0	0	0	0	0	6	11	55
	Minor	33	0	1	6	0	1	0	3	44	
NIDR	Major	0	0	0	0	0	0	3	0	3	3
	Minor	0	0	0	0	0	0	0	0	0	
NIMH	Major	1	0	0	0	0	0	0	1	2	7
	Minor	3	0	1	0	0	1	0	0	5	
NICHD	Major	5	0	1	0	0	5	0	2	13	70
	Minor	20	0	0	0	3	34	0	0	57	
NEI	Major	0	0	0	0	44	0	0	0	44	75
	Minor	0	0	0	0	30	0	0	1	31	
GRAND TOTAL		680	273	112	257	89	59	12	44		1526

Serial No. NCI-3800

TABLE 4

Number of Surgical Procedures Performed
by NCI Surgery Branch Staff
and Magnitude of the Procedures in 1971

	10 East	Other NCI	Other Institutes	Total
1963	470	159	399	1028
1964	424	215	256	895
1965	394	112	199	705
1966	410	113	218	741
1967	440	145	140	725
1968	394	205	113	712
1969	339	233	158	730
1970	301	273	230	804
1971	228	273	179	680
Major	125	72	87	284
Minor	103	201	92	396

Objective and Approach of the National Cancer Program:

Objective 5: To achieve an accurate assessment of the presence, extent, and probable course of cancer risks in population groups (including attention to pre-cancerous lesions) and of cancers in individuals alone (diagnosis) and in groups (detection) as an aid to prevention, cure, or prognosis.

Approach 4: Improve diagnostic procedures for cancer and high and low risks for localization of lesions.

Publications:

Cohen, I.K., and Ketcham, A.S.: Parotid duct translocation - Discussion of sialorrhea (Letter to the Editor), New Eng. J. Med.: 284-334, 1971.

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Catalona, W.J., Engelman, K., Ketcham, A.S., and Hammond, W.G.: Familial medullary thyroid carcinoma, pheochromocytoma and parathyroid adenoma (Sipple's Syndrome). Study of a kindred. Cancer 28: 1245-1254, 1971.

Marshall, J.R., and Henkin, R.I.: Olfactory acuity, menstrual abnormalities and oocyte status. Ann. Intern. Med. 75: 207-211, 1971.

In Press:

Morton, D.L., Glancy, D.L., Joseph, W.L., Adkins, P.C., and Johnson, R.E.: Management of radiation-induced pericarditis with effusion. Ann. Thor. Surg.

Ketcham, A.S., Hoye, R.C., and Riggle, G.C.: The laser in the surgical laboratory. Panminerva Medica.

Shimkin, P.M., Doppman, J.L., Powell, D., Marx, S.J., and Ketcham, A.S.: Demonstration of parathyroid adenomas by retrograde thyroid venography. Radiology.

Serial No. NCI-3801
1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Evaluation of Cancer Surgery as to its Effectiveness and Safety in Providing Definitive and Therapeutic Care of Cancer Patients

Previous Serial Number: Same

Principal Investigators: A.S. Ketcham, M.D., and entire Surgery Branch, NCI

Other Investigators: L.B. Thomas, M.D., S. Kakehashi, M.D., K.I. Cohen, M.D., R.E. Johnson, M.D., G.S. Johnston, M.D., and S.M. Larson, M.D.

Cooperating Units: Laboratory of Pathology, NCI; Dental Service Branch, NIDR; Experimental Therapeutics Branch, NHLI; Radiation Branch, NCI; Diagnostic Radiology Department, Department of Nuclear Medicine, Clinical Pathology Department, Nursing Department, Anesthesiology Department, Social Work Department, Rehabilitation Department, Clinical Center; NINDS.

Man Years:

Total:	8-1/2
Professional:	5
Other:	3-1/2

Project Description:

Part I. Study of the Biologic Behavior and Results of Surgical Therapy of Cancer of the Uterine Cervix

A. Surgical Treatment of Carcinoma of the Cervix

B. Scalene Nodes as Indicators of Disseminated Cancer

Part II. Study of the Biological Behavior and Results of Surgical Therapy of Cancer of the Head and Neck Regions

A. A Retrospective Computer Analysis of Patients with Head and Neck Tumors Treated Surgically at the NIH

B. Sarcomas of the Head and Neck

Part III. The Role of Bacterial Infection in Cancer Surgery

Part IV. The Evaluation of Uretero-ileostomy Following Urinary Diversion

Part V. Axillary Dissection with Preservation of the Pectoralis Major Muscle

Part VI. Extended Deltopectoral Flap Reconstruction of Anterior Oral Defects

Part VII. The Development and Evaluation of the Technique of Reconstruction of Defects of the Head and Neck Utilizing an Extended Shoulder Flap

Part VIII. Hemipelvectomy: Its Role in the Treatment of Extremity Sarcoma

A. The Study of Extremity Sarcoma

Part IX. The Surgical Treatment of Melanoma

Part X. Vascular Complications of Radical Cancer Surgery of the Pelvis and Groin

Part XI. An Improved Technique for Radical Groin Dissection

Part XII. Variation in Tumor-Doubling Time in Patients with Pulmonary Metastatic Disease

Part XIII. The Use of Coumadin Anticoagulation as an Adjunct to the Surgical Treatment of Sarcomas

Project Description:

Part I. Study of the Biologic Behavior and Results of Surgical Therapy of Cancer of the Uterine Cervix

A. Surgical Treatment of Cercinoma of the Cervix

Objectives:

To evaluate extended surgical procedures for treatment of patients with recurrent cervical cancer and for selected patients with primary carcinoma of the uterine cervix.

Methods Employed:

Approximately 2100 patients with cancer of the uterine cervix have been referred to the Surgery Branch for evaluation. From this large group

550 were admitted for inpatient evaluation, 406 of whom underwent abdominal exploration with plans for definitive surgical therapy.

Of the patients explored, 163 were not candidates for exenterative surgery because of the presence of extrapelvic lymph node metastasis, intraperitoneal spread, or disease fixed to the bony pelvis. Eighty-two patients had relatively early cancers and could be effectively managed with a radical hysterectomy and pelvic lymphadenectomy. Thirteen of these patients had small radiorecurrent lesions, the remainder had primary cervical cancer.

One hundred sixty-one patients required either partial or total pelvic exenteration for control of their disease.

Major Findings:

The major points of interest are summarized in the accompanying tables. The 5-year actuarial survival of those treated with radical hysterectomy and pelvic lymphadenectomy was 83.5%. Those patients with radiorecurrent or persistent disease survived at a rate similar to those with primary carcinoma. The presence of metastatic carcinoma in the pelvic lymph nodes adversely affected prognosis. The 5-year actuarial survival for those treated with exenteration of the pelvic viscera was 38%.

Significance to Biomedical Research and Program of the Institute:

The results obtained with extended surgical procedures are highly favorable and surpass those reported from other institutions (Annual Report on the Results of Treatment of Carcinoma of the Uterus and Vagina, 14th Volume). Extended surgical procedures are demonstrated to be effective in controlling advanced cervical cancer and are the only effective treatment modalities for patients with radiorecurrent cervical cancer. Selected individuals are offered vaginal reconstruction in an attempt to contribute to complete rehabilitation. The important ancillary studies generated by this program are detailed elsewhere in this report and include physiologic alterations following massive major surgery, dynamics of various forms of urinary diversion, the diagnostic advantages of "fine needle" cytology, and the efficacy of exfoliative cytology from urinary conduits in the early diagnosis of recurrent cancer.

Proposed Course:

The limits and the values of extended surgical procedures have been defined. Current plans call for a refinement of the diagnostic procedures to determine more specific and reliable criteria for operability and long-term cure. Although the Surgery Branch is no longer actively recruiting patients, thereby phasing out this project, significant numbers of patients with radiorecurrent disease are still being referred. These patients are included in the ancillary studies described above.

TABLE I

CARCINOMA OF THE CERVIX: ACTUARIAL 5-YEAR SURVIVAL
FOLLOWING TREATMENT WITH EXTENDED SURGICAL PROCEDURES

OPERATION PERFORMED	PRIMARY CARCINOMA PELVIC LYMPH NODES			RECURRENT CARCINOMA PELVIC LYMPH NODES		
	Positive	Negative	Total	Positive	Negative	Total
Radical Hysterectomy and Pelvic Lymphadenectomy	60.0%	91.3%	82.8% (69)*	85.7%	83.3%	84.6% (13)
Pelvic Exenterations (Partial and Total)	54.2%	52.2%	52.6% (66)	18.4%	35.6%	31.8% (95)
TOTAL (All extended surgical procedures without resection of bony pelvis)	56.4%	74.4%	67.6% (135)	30.5%	39.9%	39.1% (109)

*Numbers in parentheses represent the number of patients studied.

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TABLE II

CORRELATION OF THE MEIGS' SURGICAL PATHOLOGIC STAGING WITH SURVIVAL
OF 162 PATIENTS WITH CARCINOMA OF THE CERVIX WHO UNDERWENT PELVIC EXENTERATION

	5-YEAR CUMULATIVE PERCENT SURVIVAL		
PATHOLOGIC STAGING	PRIMARY CASES	RECURRENT CASES	ALL CASES
$A + A_o$	75% (5)*	43% (7)	60% (16)
B	56% (10)	0% (14)	33% (24)
C	31% (11)	49% (25)	44% (36)
D	60% (10)	17% (6)	44% (16)
E	43% (32)	18% (38)	30% (70)

*Numbers in parentheses represent total cases in each group.

Serial No. NCI-3801

TABLE III

PRIMARY CARCINOMA OF THE CERVIX: ACTUARIAL SURVIVAL (BY STAGE) FOLLOWING EXTENDED SURGICAL PROCEDURES

	PELVIC LYMPH NODES		
	Positive	Negative	Total
Stage I	75%	87%	84.3% (37)*
Stage II	60%	86%	80.0% (42)
Stage III	50%	75%	60.0% (10)
Stage IV	50%	32%	39.5% (37)

*Numbers in parentheses represent the number of patients studied.

Project Description:

Part I. Study of the Biologic Behavior and Results of Surgical Therapy of Cancer of the Uterine Cervix

B. Scalene Nodes as Indicators of Disseminated Cancer

Objectives:

To determine the instance of tumor dissemination, from primary pelvic cancer, to the scalene node bearing areas.

Methods Employed:

All patients with biopsy proven cancer of the structures of the female pelvis who are candidates for definitive curative surgery will have left scalene node biopsy. The procedure is carried out only in the absence of distal tumor spread. A generous pad of lymph node bearing tissue from the left subclavicular fossa is obtained from microscopic analysis. Using local anesthesia, this minor operative procedure can be carried out without complications.

Major Findings:

Sixty-nine patients with carcinoma of the cervix whose preoperative evaluation revealed no evidence of cancer spread beyond the confines of the pelvis and who were candidates for surgery had scalene lymph pad tissue removal. Eight of these patients were found to have unsuspected metastases to one of the lymph nodes in the subclavicular fossa. None of these patients had any evidence of pulmonary tumor or other metastases. They were, therefore, deemed incurable by surgery and a radical procedure avoided which would only have been palliative.

Significance to Biomedical Research and Program of the Institute:

A significant number of patients developed metastatic disease at such a time interval following primary tumor treatment that there is the suggestion that the metastases were present preoperatively. No matter how complete and extensive the pretreatment evaluation, the surgeon must face this distressing situation only too often. The identification of metastatic disease from pelvic carcinoma to unsuspected anatomical areas adds another preoperative modality of determining what patient may be free of cancer metastases at the time of definitive surgery.

Proposed Course:

Continue study of all patients admitted for treatment of primary pelvic disease to rule out previously unrecognized metastases.

Project Description:

Part II. Study of the Biological Behavior and Results of Surgical Therapy of Cancer of the Head and Neck Regions

Objectives:

1. To determine the feasibility of an aggressive surgical approach to irradiation or surgical recurrent malignancies of the head and neck areas.
2. An evaluation of adjunctive chemotherapy used before surgery and its long-term intermittent use after surgery.
3. To determine the effectiveness of a combined intracranial facial approach to cancer of the paranasal sinuses.
4. To determine the prevalence, site of origin, and rationale of treatment of unknown primary disease metastatic to head and neck areas.
5. Evaluation of new methods of surgical approach and reconstruction in surgical treatment of head and neck cancer.
6. The clinicopathological correlation of epidermoid carcinoma of the head and neck area.

Methods Employed:

Following extensive preoperative clinical, pathological, and roentgenological studies, standard and new radical surgical procedures are carried out.

(a) In patients with Stage II, III, and IV epidermoid cancers of the oral cavity treatment with methotrexate will be used for 2 to 3 weeks before surgery, and after surgery will be used on a long-term intermittent basis. Surgery will involve wide resection of the primary tumor with unilateral or bilateral neck dissection. An effort will be made to increase survival and decrease the incidence of local recurrence with this combined chemotherapy and surgery approach.

(b) In patients having paranasal sinus cancer, either primary or recurrent which suggests roentgenographically or clinically that the cribriform plate will be removed at the time of surgery, our approach will be from an intracranial and combined facial surgical incision which will permit en bloc removal of the tumor and at the same time afford protection of the surrounding vital structures.

(c) In patients requiring surgery to control recurrent disease following previous therapy, treatment will be combined pre- and postoperatively with a chemotherapy approach. During our continuing studies for inpatients with head and neck cancer some 70-80% of the patients screened for admission have been failures with previous therapy, usually irradiation. The actual incidence of patients admitted and treated who have been previous failures is 71%. By using chemotherapy before and after surgery it is anticipated that an improvement in survival results will be realized.

(d) In patients requiring large resection for treatment of their cancer immediate plastic reconstruction will be carried out using flaps of tissue from the pectoral or shoulder areas. These flaps will be used singly or in combination at the time of the resection.

Major Findings:

Over the past year 15 patients have been entered on the combined methotrexate and surgery protocol. No complications have been encountered. The majority of the patients have demonstrated a marked reduction in the size of their tumor and metastatic neck nodes during the 2 week pre-surgery treatment period. There has been no evidence of recurrent disease developing during the post-surgery treatment period.

The combined intracranial facial operation for cancer of the paranasal sinuses has been performed in 48 patients to date. A review of the results revealed that of the patients treated, 19 were dead and 29 were living for a time interval of 4 months to 11 years follow-up. The operation has been extended to include patients showing some involvement of the cerebral meninges. All of the patients who have thus far been approached in this manner have had extensive disease and have not been amenable to treatment in any other fashion. Any survival at all is indeed promising, but more patients and more time is necessary before the procedure can be recommended for routine use. The advantages of the combined procedure are that it involves (1) evaluation of intracranial disease, (2) protection of the brain, (3) avoidance of spinal fluid fistulas, (4) more adequate hemostasis, (5) facilitation of resection, and (6) en bloc resection.

Unknown primary lesions with neck metastases is not a frequent occurrence and accumulation of a series of significant numbers is difficult. The eventual discovery of the primary lesion has usually been in the nasopharyngeal sinus area, but 3 cases have been from the thyroid and 1 from the kidney. In the past year, 6 new cases have been admitted to this study.

Significance to Biomedical Research and Program of the Institute:

These locally resistant, usually recurrent head and neck lesions have long been a problem to oncologists. We are attempting to correlate aggressive surgical exploration of these lesions with survival and the mode of tumor dissemination. Careful environmental background reviews reflect the close relationship between the Surgery Branch and the Dental Prosthetic Unit allowing a comprehensive evaluation of the indications for plastic reconstruction of facial deformities and evaluation of newer prosthetic materials.

Proposed Course:

The extent of surgical excision cannot be much increased in its scope beyond that which we are now able to perform successfully. More patients are necessary before the combined cranial-facial approach to paranasal sinus cancer can be recommended for routine use. Both procedures are based on

sound experimental and clinical observations and final evaluation should prove rewarding. Pilot studies are underway to determine the feasibility of intensive preoperative medication in patients with disease which has locally extended beyond that which can be cured by conventional means.

Project Description:

Part II. Study of the Biological Behavior and Results of Surgical Therapy of Cancer of the Head and Neck Regions

A. A Retrospective Computer Analysis of Patients with Head and Neck Tumors Treated Surgically at the NIH

Objectives:

One of the least refined areas of cancer biology concerns the ability of the physician to assign prognostic significance to laboratory and clinical data. Presently clinical and histologic staging are the only reliable criteria now utilized to predict patient survival and even with these criteria there is considerable variability. With the multiple diagnostic and evaluative tests now available, an effort is being made in this study to determine other predictive factors relative to survival in the head and neck cancer patients. Available evidence indicates the defense mechanism which might be immunologically mediated in the host might be of prime importance in achieving long-term survival from many different malignancies. There has been considerable recent interest in the prognostic significance of lymph node reactivity of patients with carcinoma of the breast. Correlative value has been ascribed to bilaterally palpable axillary nodes and the cellular structures of nodes examined. A similar study is underway with patients treated surgically for cancer of the larynx.

Since cancer of the head and neck represents a variety of disease, the patients have been assigned to traditional categories based on location of the primary tumor for the purpose of comparing our survival data with other series. The first group to be studied were those patients with cancer of the larynx and hypopharynx. Patients with tumors of the oral cavity, nasal cavity, paranasal sinuses, salivary glands, skin, and unknown primary sites will be studied subsequently.

Methods Employed:

A detailed retrospective study of all head and neck cancer patients treated surgically at the NIH will be undertaken. To date 84 patients with cancer of the larynx have been studied. Approximately 200 clinical and histological variables extracted from the records of each patient were submitted for computer analysis using the data processing program of the cancer end results section.

Major Findings:

The majority of patients studied had either very extensive primary disease or radiation recurrent cancer. Of the 84 patients, 44 had intrinsic primary laryngeal tumors, 20 had extrinsic primary disease, and 20 patients had radiation recurrent or persistent tumors unresponsive to therapy. The involvement of cervical nodes was taken as the prognostic standard against which all other variables were compared. Intrinsic lesions with negative nodes had a 71% 5-year survival while positive nodes indicated a cumulative 5-year survival of 33%. As expected, positive nodes were more likely to occur with extrinsic lesions (63% vs. 24% for intrinsic lesions) and resulted in a drop in 5-year survival from 49% for patients with negative nodes to 30% with positive nodes. Other less recognized criteria were equally significant. Of the 13 patients with cytologically positive intra-operative wound washings only 2 were alive at 5 years. The likelihood of cure was considerably greater in those patients who had either a unilateral or bilateral elective neck dissection (19 patients, 78% survival) than in those patients who had a neck dissection for clinically positive nodes (30%) or in those who had clinically negative nodes and no primary neck dissection (46%). If there was a positive intra-operative frozen section margin for which additional tissue had to be taken to assure a clear margin, the total cumulative 5-year survival dropped from 54% for those with initially clear margins to 13% for those with at least one positive frozen section margin. Of the 11 patients with microscopically positive margins on permanent section the 5-year survival was 28%. Multiple preoperative variables were likewise statistically evaluated and found to be significant in predicting ultimate survival. For example, in patients with preoperative serum albumin levels of less than 3 mgm%, the cumulative 5-year survival was 29% while those with albumin levels of 3.9 mgm% or greater had a 75% likelihood of living 5 years.

Significance to Biomedical Research and Program of the Institute:

It is apparent that there are numerous clinical tests and other less utilized variables which have prognostic significance in predicting long-term survival in patients treated surgically for cancer of the larynx. Hopefully, similar variables will be identified in other head and neck cancers as this project proceeds.

Proposed Course:

The remainder of the patients with head and neck cancer treated at the NIH will be studied retrospectively using the same computer program for data processing.

Project Description:

Part II. Study of the Biological Behavior and Results of Surgical Therapy of Cancer of the Head and Neck Regions

B. Sarcomas of the Head and Neck

Objectives:

To evaluate the experience of treating the rare, but highly lethal sarcoma of the head and neck area.

Methods Employed:

Nineteen patients with sarcomas of the head and neck area have been treated by the Surgery Branch and followed for a minimum of 4 years. Each of these patients, except for 1 child with an embryonal rhabdomyosarcoma, had large tumors which had extensively destroyed adjacent normal structures. Ten were recurrent or persistent following previous surgery or irradiation. Ten were treated by combined craniofacial resection, in 4 of whom the orbital contents were conserved.

Major Findings:

Seven patients died of their disease from 3 to 8 months postoperatively, 9 are living free of tumor, and 3 have died free of cancer, but of complicating cardiovascular disease. There were no operative deaths.

Significance to Biomedical Research and Program of the Institute:

Through an aggressive surgical approach to sarcomas of the head and neck, a 4 to 10 year cure rate of 50% may be provided even to patients with lesions arising or extending into the ethmoid sinuses. Cribriform plate resection for these large, usually recurrent lesions is carried out through a combined craniofacial approach. Although massive tissue resection is often mandatory with the truly en bloc type of resection provided by this approach, acceptable cosmetic results are obtained by preoperative reconstructive planning.

Unawareness of the propensity of these tumors to recur after marginal excision and an unwillingness to create a cosmetic deformity or attempts to cure by radiotherapy alone, or even by chemotherapy, account in great part for the low cure rates often reported. The frequent histological classification of unclassified, undifferentiated and undetermined types of biopsied sarcoma and the frequency with which diagnoses are changed suggest that the inaccuracy of specific diagnosis should not be a critical factor in determining therapy. Irradiation and chemotherapy have not generally been shown to offer advantages over surgery for sarcoma of the head and neck region.

Proposed Course:

Continued evaluation of all modalities of treatment for sarcomas of head and neck will be carried out. Attempts will be made to indoctrinate the medical community about the increased salvage that may be obtained in these patients by first, early recognition and second, aggressive therapy.

Project Description:

Part III. The Role of Bacterial Infection in Cancer Surgery

Objectives:

To further substantiate findings in the previous years of the evaluation of the operative wound infection rate and its relation to the "prophylactic" use of antibiotics following extensive cancer surgery.

Methods Employed:

In the past year the immediate admission and repeated preoperative culturing of all patients' nose, skin, and tumor allowed patients to be classified into either carrier or noncarrier status of hemolytic staphylococcus aureus coagulase-positive organisms. By bacteriophage typing, through the cooperation of the Clinical Pathology Department, a correlation could be drawn between the patient's preoperatively carried organism and that carried postoperatively, either asymptomatically or related to a postoperative infection. In previously reported studies patients undergoing radical cancer surgery had, on a double blind basis, either 10 days of Chloromycetin postoperatively or a placebo. A subsequent study entailed the use of 7 days of Chloromycetin postoperatively in all patients, while on a double blind basis the patients received preoperatively either 3 days of placebo or 3 days of antibiotic. The current protocol utilized intravenous antibiotics before, during, and 7 days following the operation. Following 2 separate studies, which have been reported, antibiotic therapy has been used prophylactically in a therapeutic manner on all patients undergoing cancer therapy. These patients have been carefully observed for wound infection and any possible correlation to their "carrier status" or pathogenic organisms.

At the present time, close infectious surveillance is carried out but without a formalized protocol which calls for specific culturing of more treatment regimes

Major Findings:

The previous annual reports detail the specific results of both double blind antibiotic studies. In brief, the infectious complication rate following extensive cancer surgery was lowered from 54% to 14% by the use of pre- and postoperative antibiotics rather than placebo. An 8% infection rate occurred when the intraoperative use of antibiotics was employed in addition to pre- and postoperative coverage.

The overall results for the last 16 years can be seen in the accompanying table. The 1960 rate was elevated to 32% in that this was the year of the original study, whereas 1959 was lowered somewhat because of the discontinuance of the use of penicillin and streptomycin and the selection of antibiotics was left to the discretion of the surgeon.

16 YEAR SUMMARY OF INFECTION RATES AND
STAPHYLOCOCCAL INCIDENCE FOLLOWING EXTENSIVE CANCER SURGERY

Year	Extensive cancer operations	Infection rate per cent	Per cent of total infections that were staphylococcal
1956....	128	28	35
1957....	139	31	42
1958....	145	30	59
1959....	130	22	69
1960....	144	32	74
1961....	115	13	73
1962....	132	10	72
1963....	137	15	70
1964....	131	7	51
1965....	132	6	36
1966....	141	5	22
1967....	128	6	17
1968....	209	8	14
1969....	217	10	8
1970....	132	11	7
1971....	197	13	4

During the past 16 years, 2605 extensive cancer operations have been performed by the Surgery Branch of the National Cancer Institute. During the early part of this study the infectious complications ranged from 22% to 31% during a time when penicillin and streptomycin were used somewhat routinely at the discretion of the surgeon. The results of a double blind antibiotic-placebo study revealed an infection rate of 54% when a placebo was used and 14% when routine 10 day postoperative antibiotic therapy was instituted. A subsequent double blind study showed that by giving drug for 3 days preoperatively and 7 days postoperatively antibiotic therapy resulted in a 17% rate of infectious complications. With continued use of antibiotic prophylaxis, while continuing the same type of extended operative procedures, the infection rate was 10% and 15%. Chloramphenicol, 2 gm. every 6 hours, was the antibiotic used and there were no significant complications referable to drug toxicity and only 5 severe infectious complications referable to loss of staphylococcal sensitivity to the drug.

Over the past years the percentage of total infections that were hemolytic staphylococcus aureus DNA-ase positive in origin progressed from 35% in 1956 to 70-75% in 1961-64, and dropped to 36% in 1965 and 14% in 1968 and was

4% in 1971. At the same time, the incidence of patients who were carriers of staphylococci on admission to the hospital was 22% and has remained at that approximate level. In the past, patients who carried staphylococci into the operating room, usually asymptomatically, ran at 38% increased rate of infection over noncarriers. Ninety-three percent of these patients who subsequently become infected as an operative complication will become infected with the same phage type organism that they carried into the operating room. This suggests that self-infection may play as important a role in infectious complications as does cross-contamination. Of greater importance at this time is the alarming increase in the complications associated with Pseudomonas and Klebsiella and E. Coli infections. So frequently death is the result of a septicemia from these organisms. They are much more of a problem today, both in prevalence and in morbidity and mortality than the staphylococcus. The gradual increase in wound infections which we are again noting, from a low of 5% in 1966 and now 13% in 1971, is in part due to newly emergent organisms which have in the past been uncommon as a threat. In addition, the trend on the part of the resident staff, who are from general surgical residencies without significant cancer orientation, is to use antibiotics rather sparingly. This is a procedure well documented in general surgical cases in contrast to our experience citing the need for "prophylactic" antibiotics in cancer patients.

During the past 5 years, 12 to 24 hours of preoperative antibiotics have been used routinely along with continuous intraoperative antibiotic drugs and 3 to 7 days of postoperative therapy. The overall infectious experiences of patients undergoing very radical cancer surgery is seen in the next table.

POSTOPERATIVE INFECTIOUS COMPLICATION RATE
FOLLOWING DIFFERENT MODES OF ANTIBIOTIC THERAPY

	# of Pts.	Infections	Rate
Antibiotic used at discretion of surgeon	704	197	28%
No antibiotic therapy	87	46	53%
Postoperative therapy	332	66	20%
Pre- & postoperative therapy	288	40	14%
Pre-, post-, & intraoperative therapy	802	88	11%
In 1971 no specific antibiotic routine was used by the staff of surgeons	680	88	13%

Significance to Biomedical Research and Program of the Institute:

When postoperative morbidity and mortality are increased due to complications related not directly to the cancer but due to infection, the overall evaluation of surgery and its place in cancer therapy is markedly altered. Bringing an alarming infection rate under relative control has decreased morbidity and mortality as indicated in the previous paragraphs. In a hospital such as this where rather elaborate facilities are available for close patient surveillance, studies such as this can be done which can be of significant value to the practicing surgeon. The fact that the total infectious complication rate seems to be rising may be related to the reluctance on the part of the clinician to use Chloramphenicol which has been shown over the years to be the drug to which the hospital organisms are most susceptible. Because of the medicolegal aspects related to Chloramphenicol administration, the resident staff use this antibiotic only on direct insistence on the part of the senior staff. The continued decrease in the staphylococcal infection complication rate is consistent with findings in most hospitals throughout the country.

Proposed Course:

The indications for "prophylactic" antibiotic usage when carrying out the present clinical investigative studies within the Surgery Branch are unequivocal. Close surveillance of carrier patients and personal carriers within the hospital is being continued. It is anticipated that continuation of the surveillance program will not only lessen the so-called epidemic infectious outbreaks occasionally seen on all surgical wards but will allow more rapid and adequate control when such does occur. It is proposed that the critical period of antibiotic coverage is at the time of the operative procedure itself and that constant infusion and drug level maintenance of antibiotics during this period is mandatory in cancer surgery.

Project Description:

Part IV. The Evaluation of Uretero-ileostomy Following Urinary Diversion

Objectives:

Despite the wide acceptance of the ileal conduit, it is recognized that patients following this procedure have a significant incidence of complications related to the upper urinary tract. For this reason, such high risk patients have required constant surveillance of the urinary transport system. This is usually monitored by the frequent performance of excretory urography. Repeated use of such radiographic procedure carries with it the inherent risks associated with cumulative irradiation as well as idiosyncratic or allergic reactions. Since patients undergoing conduit diversions for benign and malignant disease can now expect a relatively long period of survival, a safe, accurate procedure to regularly evaluate the status of urinary transport system would be useful. The radioisotope renogram appears to satisfy these criteria, although its accuracy has not been formally tested in patients with

ileal conduit. The present study was designed to evaluate if the radioisotope renogram was as reliable as the excretory urogram in detecting urinary tract obstruction in patients with ileal conduit diversion.

Methods Employed:

Patients who have undergone total pelvic exenteration and ileal conduit urinary diversion for cancer of the uterine cervix at the Surgery Branch of the National Cancer Institute will be studied. Renograms and scintiphotos will be obtained using an Anger scintillation camera. Prior to the procedure each patient will receive 10 cc/kg of water by mouth which should assure a urine flow of 1.5 to 2 cc/min. All renograms and scintiphotos will then be correlated with the patient's clinical state and her excretory urogram.

Major Findings:

The first important findings concerned the proper position to perform renography in patients following urinary diversion. It was discovered that if the customary supine position was utilized for renography, a significant number of patients had an obstructive renogram pattern. If, however, the renogram was repeated in the sitting position, the pattern obtained was either normal or corresponded to the patient's excretory urogram. After the initial 15 patients had been studied in both the supine and the sitting position, all other renograms were obtained in the sitting position. An excellent correlation existed between the degree of pyelectasis and/or caliectasis and the renogram as measured by the time from injection of the isotope to the point of maximum count rate over the kidney (T-max). Although a significant difference existed between T-max values of normal appearing kidneys and those with minimal dilatation, there was some overlap between these groups. Since ureterectasis alone, and some renal units with minimal hydronephrosis, were not associated with an abnormal T-max, the anatomic abnormalities seen by urography may not be indicative of a functional obstruction. All patients with moderate or marked hydronephrosis had abnormal renograms.

Significance to Biomedical Research and Program of the Institute:

The accuracy, safety, and convenience of renography make it ideally suited for the long-term monitoring of patients with ileal conduit urinary diversion. We feel that patients with ileal conduit urinary diversion can be safely followed for long periods of time with repeated renograms rather than every 6 months to 1 year excretory urograms. In addition to the renogram the patient should have the customary studies for urinary function, urine culture, as well as a KUB x-ray to rule out calculus formation.

Proposed Course:

Patients following ileal conduit urinary diversion will continue to have renography performed and additional patients added to the study to determine the accuracy of this procedure.

Project Description:

Part V. Axillary Dissection with Preservation of the Pectoralis Major Muscle

Objectives:

Because of the severe disability resulting from the currently used techniques of axillary dissection, a reassessment of the operation was undertaken. Heretofore, two techniques of gaining access to the axilla were used, either excision of the muscle, which greatly weakens the upper extremity, or transection and resuture of the muscle, which partially devascularizes and denervates the muscle; it also requires prolonged immobilization after the operation and often results in residual limitation of motion and weakness of the extremity.

Methods Employed:

To devise a technique of access to the axilla without transection of the pectoralis major muscle, experiments were conducted using normal volunteers, cadavers, and finally in patients having axillary dissections. It was found that a semilateral position of the patient with the arm adducted across the chest proved to give adequate exposure of the axilla contents without section of the muscle and without compromise of the goals of the operation. This unique position of the patient in turn required the development of a new technique of executing the operation.

Major Findings:

Thus far, axillary dissection utilizing this position and technique has been completed in 41 patients. With the exception of 2 in whom partial resection of the innovation of the muscle was necessary because of bulky metastatic tumor deposits, the neurovascular bundle to the pectoralis major was preserved. The average postoperative follow-up of the patients is 24 months at present. In spite of the fact that 20 patients had axillary lymph nodes involved with metastatic tumor and in 11 of these the tumor had extended through the node capsule into the axillary areolar tissue, no recurrences have developed. All patients have had per primum healing of their wounds and have regained full motion of the extremity. In addition, edema of the upper extremity commonly seen after the conventional technique of axillary dissection utilizing excision of the pectoralis major muscle has not occurred in these patients.

Significance to Biomedical Research and Program of the Institute:

Resection of the axillary lymph nodes is an established aspect of the treatment of tumors of the extremity and adjacent chest. The technique of the operation described has proved to give a superior cosmetic and functional result in our patients and has been of particular benefit in those patients who have required bilateral axillary dissections, most of whom were male laborers.

Proposed Course:

A detailed illustrated description of the operation has been published. The major cosmetic and functional advantages offered by this approach to axillary dissection suggest that it will be adopted as a standard technique.

Project Description:

Part VI. Extended Deltopectoral Flap Reconstruction of Anterior Oral Defects

Objectives:

To develop a direct, easily accomplished, and reliable means of reconstruction of defects of the chin, anterior floor of mouth, and tongue that offers a superior cosmetic and functional result to that achieved with conventional techniques of reconstruction.

Methods Employed:

To date, no satisfactory means of reconstructing defects in the chin, anterior floor of mouth, and tongue has been devised. The cosmetic deformity resulting from resection of massive defects in this region and the functional disability, e.g., continuous drool, impairment of speech, prevention of mastication, and pulmonary aspiration of saliva led to an assessment of this heretofore unsolved problem. A flap having its base in the sternal region and extending to a tip in the posterior axillary fold was developed for use in the reconstruction of these defects. The flap has been perfected so it can be raised and rotated without loss of viability of the tip and with minimum inconvenience to the patient.

Major Findings:

The extended deltopectoral flap has been utilized in 8 patients thus far, including 3 who had virtually complete loss of the free tongue. It has allowed complete restoration of speech and swallowing in each case. The technique represents the first cosmetically and functionally satisfactory means of restoration of tissue in such defects.

Significance to Biomedical Research and Program of the Institute:

Impairment of function and the distortion of the appearance of patients having massive resections of tumors involving the chin and anterior tongue region has constantly presented a challenge to the members of the Surgery Branch as well as head and neck surgeons elsewhere. The use of this technique of reconstruction will markedly shorten the hospitalization time of these patients following removal of such malignancies, and allow a rapid and more complete return to their community with elimination of the nursing care required heretofore after such operations.

Proposed Course:

Improvements in the technique of reconstruction utilizing this flap will be made so as to allow its use both at the time of resection of the primary tumor and in those patients who had resection of the tumor at sometime in the past and have residual, functional and cosmetic deficits. In addition, this flap has been used in combination with the extended shoulder flap in several patients who had full thickness losses of the face that would have been virtually impossible to construct with currently available techniques. Further improvements in the technique of reconstruction with such flaps should relieve the reticence of the surgeon to resect massive malignancies of the head and neck region which, though technically feasible, would create distortions of appearance and function that would deter the patient from accepting the operation.

Project Description:

Part VII. The Development and Evaluation of the Technique of Reconstruction of Defects of the Head and Neck Utilizing an Extended Shoulder Flap

Objectives:

With the premise that a major deterrent to the performance of radical operations in the head and neck region is the numerous prolonged and tedious operations requiring multiple procedures, each one entailing risk of loss of the tissue used in reconstruction and lengthy hospitalizations at great expense to the patient and the medical institution, a direct, reliable, and relatively easily executed technique of reconstruction of head and neck defects with an extended shoulder flap was attempted.

Methods Employed:

After a review of the conventional methods of reconstructing head and neck defects, it appeared that a flap having its base in the occipital region would provide tissue for reconstruction of the head and neck region without the necessity of secondary migrations of the pedicle and, hence, entailing risk of loss of the tissue, especially in patients who have had prior radiation therapy to the head and neck. The conventional length shoulder flap could be used to reconstruct defects in the lateral neck region, however, hence the technique of reconstruction of a flap extending from the base of the skull onto the arm was developed. Because of the great length of this flap, its tip extends to any region of the face or neck, even to the contralateral side of the face.

Major Findings:

Thus far, 15 patients have had reconstruction of head and neck defects utilizing the extended shoulder flap. Many of these defects would have been virtually impossible to reconstruct utilizing conventional techniques. The

rapid, well-tolerated, and effective reconstruction achieved with this technique has led to its adoption as a standard method for use by the Surgery Branch.

Significance to Biomedical Research and Program of the Institute:

The massive defects in the head and neck region resulting from the treatment of massive malignancies referred to the Surgery Branch has heretofore posed a great challenge because of the numerous operations required for the cosmetic and functional rehabilitation of the patient postoperatively. The extended shoulder flap has proved to be a reliable, easily accomplished, and most rapid means of reconstructing such defects.

Proposed Course:

A detailed, illustrated description of the technique of reconstruction utilizing the extended shoulder flap has been prepared. This material was presented at a recent meeting of the Society of Head and Neck Surgeons, and published in a periodical, with a recommendation that it be adopted for use in reconstruction of most defects of the head and neck region not amenable to closure with local tissue. Other uses for this technique of reconstruction are being assessed, e.g., it has been used in combination with the extended deltopectoral flap described in another project in this report in several patients to successfully reconstruct massive full thickness losses of the face virtually impossible to satisfactorily reconstruct with conventional techniques.

Project Description:

Part VIII. Hemipelvectomy: Its Role in the Treatment of Extremity Sarcoma

Objectives:

To develop a technique of hemipelvectomy that minimizes the skill required, the operative time, and blood loss entailed.

Methods Employed:

As a result of the reluctance of surgeons to perform hemipelvectomy when indicated, because of the technical skill required in the execution of the operation, lesser procedures which lead to a higher recurrence rate and mortality rate are being employed on a widespread basis. In an effort to improve the survival rate with such patients, we have recently completed a study of the operation in cadavers and living patients and devised a technique that is within the skills of the average general and orthopedic surgeon.

Major Findings:

The conclusion of an assessment of the problem was that the most difficult aspect of hemipelvectomy as conventionally performed was the deep pelvic

dissection. We then found that the relatively limited exposure to this anatomically complex and vascular area afforded when the operation is performed with the patient in the supine position is obviated with the patient in the lateral decubitus position. The addition of the Trendelenburg position then afforded additional exposure because of the gravitational shift of the viscera into the upper abdomen. In addition, simultaneous access to the anterior and posterior aspects of the pelvis resulting from this patient position, as well as the perineum when the extremity was elevated, allowed the rapid execution of the operation, particularly in those instances in which uncontrollable bleeding was encountered during the deep pelvic dissection. After studies in cadavers suggested that these concepts should be assessed in living patients, the operation was so utilized with such great success that it was adopted as the standard technique by the Surgery Branch.

Significance to Biomedical Research and Program of the Institute:

The alarming number of lower extremity malignancies referred to this Institute after recurrence with inadequate operation because of the reluctance of the referring physician to perform hemipelvectomy indicates the need for an easily mastered technique for the operation. The technique developed should have a favorable effect on the surgeon's willingness to perform this operation as the initial procedure, thus giving the patient the maximum opportunity for cure. In addition, the utilization of this technique by the Surgery Branch has shortened the operative time and the blood loss entailed in the operation, and allowed the safer performance of these operations in relatively poor risk patients.

Proposed Course:

A detailed and illustrated disclosure of the technique for publication in a surgical periodical is in progress. In addition, a motion picture depicting the advantages of the operation as well as a detailed step by step conduct of the operation in a patient has been completed and submitted to the Film Library of the American College of Surgeons to make it available for distribution to general and orthopedic surgeons.

Project Description:

Part VIII. Hemipelvectomy: Its Role in the Treatment of Extremity Sarcoma

A. The Study of Extremity Sarcoma

Objectives:

A review of patients operated on by the Surgery Branch of the National Cancer Institute for sarcomas of bone as well as soft tissue sarcomas primarily involving the trunk and extremities was undertaken. The questions of survival statistics, incidence of regional node metastases, and the incidence of

local recurrence following surgery were of particular interest. Also of interest were the results of therapy at the National Cancer Institute as compared to the published results country-wide.

Methods Employed:

Case records of patients operated on by the Surgery Branch from 1953 until December 31, 1968, were reviewed. Patients studied were those who underwent definitive surgery at this institution.

Major Findings:

Sixty-two patient records were reviewed in detail. The survival statistics for 2 years and greater demonstrated the following: rhabdomyosarcomas, 3 of 8; liposarcoma, 2 of 4; undifferentiated sarcomas, 1 of 4; fibrosarcomas, 7 of 14; chondrosarcomas, 8 of 11; synovial sarcomas, 1 of 1; mesenchymal sarcomas, 1 of 2; osteogenic sarcomas, 2 of 16 (1 of these 2 was a parosteal sarcoma); leiomyosarcoma, 1 of 1.

This gave an overall survival rate of 26 out of 62 patients.

In this 15 year period there were 21 hemipelvectomies performed and 9 interscapular thoracic amputations performed. The mortality from this extensive surgery was minimal, only 1 patient of these 62 died in the postoperative period and that was a patient who died of renal failure after a combined total pelvic exenteration-hemipelvectomy.

There were 4 local recurrences, 1 following radical local excision, 2 following interscapulothoracic amputation, and 1 following hemipelvectomy. These local recurrences were all in the patients who had massive local lesions.

Significance to Biomedical Research and Program of the Institute:

Survival statistics and local recurrence rates were comparable to published reports. Of particular importance was documentation of the extremely poor outlook for the patient with an osteogenic sarcoma, with the survival being 10% or less at this institution. The other common lesion treated here with a poor prognosis was fibrosarcoma, with a 50% survival rate greater than 2 years. These data have suggested that we can select patients on the basis of their histological diagnosis and the size of the lesion to represent very poor risk patients and include them in some type of an adjuvant protocol. At present only the patients with metastatic cancer have been treated by immunotherapy, but certainly patients with an expected survival of only 10% would be an excellent group to attack aggressively with immunotherapy. Since osteogenic sarcomas presumably do contain a common tumor specific transplantation antigen aggressive immunotherapy seems indicated. In addition, patients with osteogenic sarcomas or large soft tissue sarcomas would be acceptable for aggressive adjuvant chemotherapeutic protocol.

Proposed Course:

The patients who have been operated on since January 1, 1969, will be reviewed to keep the study up to date. This data is being made available to the National Sarcoma Task Force for country-wide evaluation of patients with soft tissue sarcomas. Further evaluation of rehabilitation of patients who have been amputated is being undertaken to better evaluate patients preoperatively and to assist them in postoperative convalescence. The data is being looked at closely from the standpoint of seeing with which tumors we can avoid radical amputative surgery or particularly in patients whose prognosis is grim.

Project Description:

Part IX. The Surgical Treatment of Melanoma

Objectives:

To determine the role of relatively standardized but aggressive surgical approach to malignant melanoma.

Methods Employed:

All cutaneous lesions are excised with a minimum margin of 4 cm. of grossly normal skin. Skin grafting is used for closure in 99% of these local excisions. En bloc primary resection encompasses excision of fascia and the deep subcutaneous tissue. In continuity resection of the draining lymph nodes is carried out if feasible. When midline lesions are encountered, bilateral lymphatic drainage sites are removed. Amputative surgery is not ordinarily indicated except in digital lesions. Melanomas on the distal extremity are not necessarily resected in continuity with groin or axilla. Radical deep groin dissection is no longer carried out in continuity with the superficial groin unless a superficial groin node is confirmed positive by frozen section examination. The deep groin node dissection is now thought to be best performed via transabdominal approach at a second stage, giving less postoperative morbidity in the distress of leg lymphedema.

Major Findings:

This is a long-term program treating a very distressing type of cancer. Regarding local control, the recurrence rate is less than 7%. Failure to control distant metastatic disease, however, is the primary cause of overall mortality of 60%. Studies are in progress to evaluate these patients more adequately preoperatively as well as attempts to approach their metastatic disease, such as with endolymphatic radioactive isotopes.

Significance to Biomedical Research and Program of the Institute:

The bizarre behavior of this tumor makes it one of the more poorly understood cancers. Melanoma from these patients is divided into several experimental

laboratory protocols and attempts to stimulate immunologic host responses in these patients is being done using BCG-induced hypersensitivity.

Proposed Course:

This ongoing long-term program involves the cooperative efforts of groups of laboratory investigations. Patients developing recurrent or metastatic disease will be considered for experimental immunotherapy. The charts of 240 melanoma patients are now being reviewed for consideration of the following questions:

1. What influence does the anatomic site of malignant melanoma have on subsequent survival following therapy?
2. What influence does sex have on survival following therapy?
3. Has prophylactic regional node dissection proved beneficial in the treatment of malignant melanoma?
4. Has therapeutic node dissection proved to be beneficial?
5. What factors relating to the primary lesion are important determinants of survival (histology, depth of invasion, size of lesion, etc.)?
6. To what extent has regional node involvement with melanoma influenced survival?
7. Have other forms of therapy utilized in the Surgery Branch provided significant benefit to patients?
8. What complications have followed surgical procedures? To what extent are they seen?
9. Are these particular groups of patients worthy of special scrutiny because of unusual circumstances relating to the course of the disease (e.g., familial melanoma, disease associated with melanoma, prepubertal melanoma, multiple primary melanomas, etc.)?

Project Description:

Part X. Vascular Complications of Radical Cancer Surgery of the Pelvis and Groin

Objectives:

To review major vascular complications arising during the operative treatment of cancer by radical pelvic or groin surgery, and to derive some principles to be applied in the future for similar situations.

Methods Employed:

A series of cases in which major vascular complications occurred have been critically reviewed and literature regarding this sort of problem has been surveyed. Follow-up studies on the patients involved have been carried out insofar as availability of patient material permits.

Major Findings:

Ten patients with major vascular complications were reviewed. The conclusions derived from this review were several. The first was that an aggressive approach to these vascular problems is usually the most satisfactory approach. The second is that the early use of contrast angiography (either venous or arterial) in the assessment of the disease is an important adjunct to solution of the particular problem. The third conclusion is that a major source of postexenterative venous complications is damage to major vein walls at the time of surgery. It is suggested that in such instances prophylactic caval plication be considered at the time of total pelvic exenteration. The fourth conclusion is that in the late follow-up of patients with leg edema following radical pelvic or groin surgery benefit may ensue by performing appropriate angiographic studies and vascular reconstructive procedures as indicated thereby.

Significance to Biomedical Research and Program of the Institute:

As a greater number of people are undergoing radical cancer surgery in the pelvis or groin this is hoped that the data obtained from these studies will lead to a decreasing incidence of complications and a more satisfactory program for treating them when they do arise. The vascular complications of this particular kind of surgery have not been seriously scrutinized in the past and this assessment provides helpful guidelines for further more detailed evaluation of this problem in this type of patient.

Proposed Course:

Publication of data already obtained. Ongoing surveillance of case material with appropriate studies to substantiate in a prospective fashion the conclusions thus far reached from the retrospective study.

Project Description:

Part XI. An Improved Technique for Radical Groin Dissection

Objectives:

Standard techniques of radical groin dissection entail significant immediate and long-term morbidity. The immediate morbidity is primarily due to necrosis of the skin flaps leading to infection of the operative wound, thrombosis of the femoral vessels, and thromboembolism. Prolonged hospital stays occur in approximately 1/2 of patients having this operation. The long-term morbidity consists of persistent and incapacitating lower extremity edema with limitation of motion and skin ulceration. This study was undertaken to develop an improved technique of radical groin dissection which would obviate these morbidities.

Methods Employed:

Through a trial of all existing techniques and study of the anatomical factors involved in radical groin dissection, a technique was devised that utilizes two incisions. An oblique incision paralleling the inguinal crease is used to dissect the superficial inguinal lymph nodes. A second, midline incision is used to dissect the pelvic lymph nodes. In this technique, the circumflex iliac and inferior epigastric vessels are preserved.

Major Findings:

Using the technique described, thus far 22 patients have had radical groin dissections. Fourteen of these were unilateral and 8 were bilateral radical groin dissections. The immediate morbidity due to flap necrosis has been minimal, and in no case has delayed the ambulation of the patient. The long-term result has likewise been gratifying. None of these patients has developed extremity edema or morbid skin changes.

Significance to Biomedical Research and Program of the Institute:

An important contribution to the treatment of cancer patients would be the reduction of the morbidity associated with radical groin dissection. This operative technique appears to have achieved this goal. This is reflected in decreased postoperative hospitalization time and concomitant or rapid return to preoperative functional capacity of the patients who had radical groin dissection by this operative approach.

Proposed Course:

Continued study of this technique is in progress with an attempt to further improve it and adopt it for all situations requiring radical groin dissection.

Project Description:

Part XII. Variation in Tumor-Doubling Time in Patients with Pulmonary Metastatic Disease

Objectives:

Recent experience has shown that the pulmonary doubling time (TDT) is an easily measurable, uniform, and accurate method for judging the progression of pulmonary metastatic disease in cancer patients. Previous studies by the Surgery Branch have shown a correlation between the pulmonary doubling time and survival following resection of pulmonary metastases. Although most patients have been easily categorized relative to pulmonary metastatic TDT, patients with widely varying TDT have been encountered. This study ascertains the effect of this variable on their prognosis following pulmonary metastatic tumor resection.

Methods Employed:

The method of determining pulmonary doubling time developed on this service entails examination of chest x-rays made at two time intervals and the estimation of doubling time by a graph which utilizes the two diameters obtained from measure of the tumors on x-ray. Resection of the pulmonary deposit was done after determination of pulmonary doubling time. All patients were then followed to determine effect of tumor resection on survival.

Major Findings:

Forty-five patients with multiple pulmonary metastatic foci have their TDT measured. These groups consist of patients with TDT of less than 20 days, those with TDT of 21-40 days, and those with TDT of over 40 days. In 37 patients, the calculated TDT's for each individual were similar and allowed the patient to be placed in particular groups based on their growth rate. In 8 patients, there was a particularly wide variation in TDT such that placement of these individuals into a particular category was impossible. These patients all underwent resection of their pulmonary metastases. All except 1 had at least 1 metastatic nodule that fell in the slow-growing category (more than 40 days). All patients developed new metastases after pulmonary resection. This suggests that prognosis appears to be determined by the tumors with faster TDT rather than the slower ones.

Significance to Biomedical Research and Program of the Institute:

Both significant palliation and long-term curative benefits have been achieved in patients by resection of pulmonary metastases. It is important to clearly delineate which patients can be expected to benefit from this type of surgery so that the number of unsuccessful operations will be reduced to a minimum. This study shows that the course of patients that have widely varying TDT's will be biologically like those who have short TDT's after pulmonary resection. This finding indicates that surgery in these patients will be attended by a high recurrence rate.

Proposed Course:

Continued study on patients with varying pulmonary TDT's will be made to further delineate the biological variables affecting their prognosis.

Project Description:

Part XIII. The Use of Coumadin Anticoagulation as an Adjunct to the Surgical Treatment of Sarcomas

Objectives:

To see if anticoagulation preoperatively, intraoperatively, and postoperatively following surgery for sarcoma of the extremities can improve the poor survival rates in this disease.

Methods Employed:

Patients with histologically proven sarcoma which is felt to be capable of cure by amputation, hip disarticulation, or hemipelvectomy will be admitted to the study. Evidence of metastatic disease or contraindication to anticoagulation excludes the patient.

The more sensitive measurement of the level of plasma coagulated factors, namely the prothrombin-proconvertin (P & P), is utilized in order to control the level of anticoagulation precisely. This level of anticoagulation is maintained during surgery and also postoperatively.

The patients must be on stable anticoagulation for 72 hours prior to surgery with the prothrombin time 2 to 2-1/2 times control and a P & P 10-25% of normal. In the initial phases of the study, we have elected not to include patients undergoing hemipelvectomy until we have sufficient experience with the anticoagulation technique. In addition, patients undergoing hemipelvectomy or forequarter amputation combined with other major surgical procedures will not be included. However, groin or axillary node dissections in combination with amputative surgery does not exclude patients from the study.

Major Findings:

Eleven patients have been included in the protocol thus far. Four are dead of their disease, 6 are alive, and 1 is alive with metastases. However, in 4 patients less than 24 months have elapsed since surgery. Five patients treated with amputation were anticoagulated but the protocol had to be abandoned due to bleeding into the stump which required termination of anticoagulation.

Significance to Biomedical Research and Program of the Institute:

A very high mortality rate continues to occur in patients with sarcomas, despite radical amputative surgery. For this reason, effective adjunctive therapy must be searched for in this disease. It is hoped that anticoagulation will improve the results of therapy in this group of patients and even more importantly lead to an understanding of the mechanisms of tumor dissemination common to all malignancies.

Proposed Course:

Maximum efforts will be directed toward adjustment of the extent and duration of anticoagulant administration during the preoperative and intraoperative period, so as to reduce hemorrhagic complications to a minimum, yet provide as effective anticoagulation as possible during the critical operative period when dissemination of tumor cells may be expected to occur.

Objective and Approach of the National Cancer Program:

Objective 4: To prevent tumor establishment from cells already capable of forming cancers, e.g., transformed cells, cells constituting pre-cancerous tissues, and cells from primary tumors that lodge elsewhere in the body in a metastatic state, either active or dormant.

Approach 4: Interfere with the process of tumor initiation following cell transformation, including interference with development of stromal and blood vessel elements and other host responses.

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Serial No. NCI-3805
1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Experimental Studies of Mechanisms of Metastases

Previous Serial Number: Same

Principal Investigators: A.S. Ketcham, M.D.

Other Investigators: P.B. Chretien, M.D., R.C. Hoye, M.D., J.J. Ryan, M.D., P.J. Deckers, M.D., A.M. Cohen, M.D., E.V. Sugarbaker, and Hilda Wexler

Cooperating Units: Laboratory of Pathology, NCI

Man Years:

Total:	3-1/2
Professional:	1
Other:	2-1/2

Project Description:

Part I.

A. Bioassay of Circulating Murine Tumor Cells: A Comparison Between Intravenous and Intramuscular Inoculation on Tumor Incidence

B. The Fate of Circulating Methylcholanthrene Tumor Cells in Mice with Tumor Specific Immunity

C. The Effect of Tumor Immunity on the Production of Lung Tumors after Intravenous Inoculation of Antigenically Identical Tumor Cells

D. Specificity of the Facilitation of Intravascularly Inoculated Tumor Cells in Tumor Immune Hosts

E. The Effect of Serum from Tumor-Bearing Animals on the Survival and Propagation of Circulating Tumor Cells

F. The Effect of Prolonged Anticoagulation on Spontaneous Tumor Metastases

Part II.

A. The Stimulation of Dormant Cancer Cell Growth by Hepatic Trauma

B. Differences in Immunogenicity Between Primary Tumors and Their Metastases

C. Effect of Tumor Immunogenicity on the Metastatic Potential of Circulating Tumor Cells from Tumor Metastases

D. Immunity of the Autogenous Host to Viable Tumor Cells Shed from Pulmonary Metastases

Project Description:

Part I.

A. Bioassay of Circulating Murine Tumor Cells: A Comparison Between Intramuscular Inoculation on Tumor Incidence

Objectives:

To compare the relative tumor incidence after I.V. and I.M. inocultations of tumor cells in mice.

Methods Employed:

Three host tumor systems were studied: The 89th transplant generation of a methylcholanthrene sarcoma in C57BL/6N mice, the 66th generation of a spontaneous mammary adenocarcinoma in C3H/HeN mice, and the S-91 melanoma in CDF1 mice. Pooled blood was aspirated from the right heart of several tumor bearing donors that also had lung metastases. One ml. was injected into the tail vein of a normal syngeneic recipient, and 1 ml. was injected into the leg muscle of another normal mouse. Pulmonary embolic tumor growths were detected by India ink differential staining for the fibrosarcoma and adenocarcinoma. Melanoma implants were easily identified macroscopically because of their black pigmentation.

Major Findings:

In two of the three tumor groups studied, the I.V. method was more sensitive than the I.M. method in detecting the presence of circulating tumor cells. For CDF1 mice, in 13 pairs the I.V. method detected circulating tumor cells when the I.M. did not. In contrast, in only four pairs the I.V. method failed to detect cells when the I.M. did. For C3H/HeN mice, in six pairs the I.V. method detected circulating tumor cells when the I.M. did not. In contrast, in only two pairs the I.V. method failed to detect cells when the I.M. method did. For C57 mice, circulating tumor cells were similarly detected in both the I.V. and I.M. methods.

Significance to Biomedical Research and Program of the Institute:

The increased number of pulmonary tumors produced after I.V. inocula of tumor cells in comparison with the numbers of tumors produced with I.M. inoculation of the same tumor cell dose indicates the operation of factors that favor the survival and propagation of tumor cells in the circulating blood in comparison to their survival in extravascular depots. These factors may also be operative in humans with circulating tumor cells shed from a primary malignancy. An investigation and definition of the factors determining the results obtained in this experiment, therefore, may aid in the elucidation of factors that influence the development of blood-borne metastases in cancer patients.

Proposed Course:

A systemic investigation of the factors determining the selective survival of tumor cells in the circulating blood is in progress.

Project Description:

Part I.

B. The Fate of Circulating Methylcholanthrene Tumor Cells in Mice with Tumor Specific Immunity

Objectives:

To assess the effect of immunity on the survival of circulating tumor cells.

Methods Employed:

One host system was studied. An immunogenic methylcholanthrene tumor was injected into syngeneic mice, and the resulting tumor growth was then removed. The production of tumor specific immunity to these mice was shown by I.M. challenge with cell suspensions of the tumor. The effect of immunization to the tumor on circulating tumor cells was assessed by determining the number of lung tumors that resulted from I.V. injection of cell suspensions of the tumor and blood from donor mice that had circulating tumor cells.

Major Findings:

Syngeneic mice were immunized to a methylcholanthrene-induced tumor and immunity was demonstrated by the failure of cell suspensions of the tumor to grow when inoculated intramuscularly. Identical cell suspensions inoculated intravenously, however, produced pulmonary tumors in all immunized animals. Intravenous inoculations of blood containing circulating tumor cells obtained from donor mice bearing the same tumor produced identical numbers of pulmonary tumors in immunized and unimmunized animals.

Significance to Biomedical Research and Program of the Institute:

In this study, immune resistance to tumor growth as demonstrated by the failure of I.M. inocula to grow in animals previously exposed to the tumor was not reflected in a similarly heightened resistance to tumor growth where the tumor cells were inoculated I.V. In this tumor system, therefore, the ability of the tumor cells that leave the primary to survive and give rise to secondary growths is dramatically increased if they gain entrance into the circulatory system. This paradoxical increased survival and production of pulmonary embolic tumor by circulating tumor cells in hosts with tumor specific immunity may have relevance to the development of pulmonary metastasis in patients who have demonstrated specific immunity to their previously excised primary tumor. The presence of factors determining the results in this experiment in such patients would facilitate the survival of tumor cells that entered the circulatory system prior to the excision of their primary tumor.

Proposed Course:

The results of this study give evidence for the operation of serum factors that facilitate the survival and propagation of circulating tumor cells. An investigation now in progress which involves immune serum in combination with tumor cell suspensions is being used to further explore this hypothesis.

Project Description:

Part I.

C. The Effect of Tumor Immunity on the Production of Lung Tumors after Intravenous Inovulation of Antigenically Identical Tumor Cells

Objectives:

To assess and compare the survival of various doses of primary tumor cells when inoculated intravenously in immunized and non-immunized mice.

Methods Employed:

Immunogenic methylcholanthrene (MCA)-induced tumor in syngeneic C57BL/6N mice were used. Immunogenicity was determined by the classical method of inoculation intramuscularly, amputation of the tumor bearing legs and later challenge. Three tumors were shown to be highly immunogenic, one was weakly immunogenic, and another non-immunogenic. A preliminary study was conducted with one highly immunogenic tumor. Tumor cells in 1000, 500, and 100 cell doses were inoculated intravenously (I.V.) and intramuscularly (I.M.) into normal and tumor-immunized recipients. From this data, it was apparent that the 100 cell dose gives optimum differences between immunized and control animals with intravenous inoculation. Using the other four tumors, animals were inoculated intravenously with 100 cells of these four tumors two weeks after amputation of their tumor bearing legs.

Major Findings:

With the first highly immunogenic tumor studied, I.M. inoculation of immunized animals with 100, 500, and 1000 tumor cells produced a uniform 10% incidence of tumor growth. I.M. inoculation of normal animals produced increasing numbers of tumor cells with larger inoculate; 100 tumor cells produced a 15% incidence of growth and 1000 tumor cells produced an 83% incidence of tumor growth. With I.V. inoculation of normal animals, the tumor growth parallels that obtained with intramuscular inoculation: 30% of normal animals developed tumors after inoculation with 100 cells, and 90% developed tumors with the 100 cell inocula. Animals receiving I.V. inoculation of 100 cells had a 84% incidence of tumor growth and with 1000 cells, 87% of the immunized animals developed tumors. The other two highly immunogenic tumors produced a 92% and a 67% incidence of growth in immunized animals compared with a less than 40% incidence of growth in control animals. The differences between the growth in both immunized and control animals inoculated with 100 cells from the weakly immunogenic tumor, was not significant (85% in immunized animals and 60% in normal animals). With the non-immunogenic tumor, again no differences were seen: 53% of immunized animals and 33% of normal animals developed tumor after intravenous inoculation of 100 tumor cells.

Significance to Biomedical Research and Program of the Institute:

There are two major findings in this study: (1) the high percentage of immunized animals that developed lung tumors after I.V. inoculation of tumor cells than developed leg tumors at the site of I.M. challenge when inoculated with immunogenic MCA-induced tumors; and (2) the higher percentage of pretreated mice that developed lung tumors when compared with untreated mice, after I.V. inoculation with immunogenic MCA-induced tumors. A conclusion from this study is that cells from an immunogenic MCA-induced tumor which entered the circulating blood are less susceptible to immune destruction than when they are in extravascular sites; conversely, host mechanisms of tumor destruction are less effective against these tumor cells when they are in the circulatory system then when they are in the extravascular sites. A possible explanation for this phenomena is the action of a factor in the circulating blood of immune animals which facilitate the survival and production of pulmonary metastases by I.V. inoculated tumor cells. This in vivo phenomena has a striking resemblance to the in vitro serum inhibitory effect on the destruction of tumor cells by specifically sensitized lymphocytes. This phenomena has been demonstrated in experimental animal tumor systems and human cancer patients. It is, therefore, important that the phenomena accounting for the results of this experiment be further defined.

Proposed Course:

Experiments in progress explore the relative effects of immune destructive factors, both humoral and cellular, and serum inhibitor factors which determine the survival of circulating tumor cells. Attempts will be made to manipulate the tumor host to increase the effectiveness of the former and neutralize or

inhibit the effect of the latter.

Project Description:

Part I.

D. Specificity of the Facilitation of Intravascularly Inoculated Tumor Cells in Tumor Immune Hosts

Objectives:

To further define the phenomenon of increased survival and propagation of tumor cells in immune hosts when inoculated intravenously.

Methods Employed:

Previous studies with individual MCA-induced tumors showed that cells from immunogenic tumors, when inoculated intravenously, produced greater numbers of tumors in tumor immunized animals than normal animals, despite a decrease in the production of tumors in these immunized animals after I.M. inoculation of the tumor cells. In this study, three immunogenic MCA tumors were used. Animals were immunized with MCA-1 by the classical method of hind leg injection followed by amputation of a growing primary. These animals were later challenged intramuscularly and intravenously with cell suspensions of MCA-1, MCA-2 and MCA-3. Intramuscular inoculations contained 10^3 tumor cells, and intravenous inoculations contained 10^2 tumor cells. When several animals inoculated with intravenous tumor cells died as a result of pulmonary metastases 22 days after challenge, all animals were sacrificed and the number of tumor produced was determined.

Major Findings:

Intramuscular challenge of MCA-1 immunized animals with the same tumor produced no tumors in 18 animals. Conversely, challenge with MCA-2 produced 20 tumors in the immunized animals and 20 tumors in normal controls. Challenge with MCA-3 produced 18 tumors in immunized animals and 19 tumors in controls. Tumors produced after intravenous inoculation: MCA-1 challenge group - 20 in immunized, 3 in controls; MCA-2 - 16 in immunized, 5 in controls; MCA-3 - 14 in immunized, 7 in controls. The study thus shows that immunization with one MCA tumor and intramuscular challenge with the same and differing tumors demonstrates the differences in immunogenicity between individual MCA tumors as classically described. With intravenous challenge, however, an increased incidence of pulmonary tumors compared with controls was produced regardless of the combination of primary tumor and challenge tumor used.

Significance to Biomedical Research and Program of the Institute:

These studies point to factors induced by tumor immunization that facilitate the survival of circulating tumor cells. These factors appear to be non-specific

in their action. The phenomenon can be explained by the action of alpha globulins, since these immunoregulatory globulins are increased in number and quantity after immunization with MCA-induced tumors. Another possibility is that the results are due to non-specific immunosuppressive effect of antigen-antibody complexes. The similarity of the increased survival of cells when inoculated intravascularly in this tumor system and the serum-protective effect of tumor cells against destruction by immune lymphocytes demonstrated in vitro with various experimental and human tumors by other investigators emphasize the importance of further defining the factor active in this experiment and deriving means for controlling its production.

Proposed Course:

Further identification and eventual isolation of the factor active in these experiments is in progress.

Project Description:

Part I.

E. The Effect of Serum from Tumor-Bearing Animals on the Survival and Propagation of Circulating Tumor Cells

Objectives:

To determine the effect of serum from animals bearing immunogenic tumors on the production of pulmonary embolic tumors by circulating tumor cells shed from the tumor.

Methods Employed:

Pooled 2 ml. blood aliquots were aspirated from the left hearts of normal C57BL/6N mice and syngeneic mice bearing a fourth generation MCA-induced tumor and pulmonary metastases. The aliquots were centrifuged and the serum separated from the packed cells. Normal mice were inoculated I.V. with 1 ml. mixtures of .5 ml. of serum and .5 ml. of packed cells. After 42 days, the animals were killed and examined for lung tumors. The incidence of lung tumors in each group was correlated with the source of the serum and cells inoculated intravenously.

Major Findings:

A high incidence (17/14, 66%) of lung tumors were produced by the inoculation of animals with serum and cells from tumor-bearing hosts. By contrast, only 8/41 (20%) mice inoculated with a mixture of cells from tumor-bearing mice and normal serum had lung tumors and only 4/41 (10%) mice inoculated with serum from tumor-bearing mice and normal serum had lung tumors. These results show that the serum contained in the 1 ml. aliquot of blood inoculated was necessary for the production of lung tumors by the circulating tumor cells in the blood

inoculum.

Significance to Biomedical Research and Progress of the Institute:

The results of this experiment show that the induction of tumor immunity in this animal tumor system facilitates the survival and propagation of circulating tumor cells and that this effect is serum mediated. Furthermore, it shows that the serum contained in .1 ml. blood aliquots is sufficient to facilitate the survival of the circulating tumor cells in these aliquots. This in vivo effect of serum in facilitating the survival of circulating tumor cells appears to be analogous to the protective effect of immune serum on tumor cell monolayers exposed to specifically cytotoxic lymphocytes. This mounting evidence for the dominant effect of serum in facilitating the survival of tumor cells in the presence of immune cellular and humoral tumor-destructive mechanisms indicates that control of metastatic tumor production will be possible only after the development of methods that inhibit the effect of these serum factors. In addition, immunotherapy protocols must be carefully scrutinized for their effect on the production of these factors. Immunization with tumor antigen or non-specific stimulation of immune system may augment the production of these serum factors and thus neutralize the effect of the tumor-destructive agents elicited by the therapy.

Proposed Course of Project:

Fractionation of immune serum from animals bearing MCA tumors by physical and immunochemical techniques is in progress with the goal of defining the serum-mediated factor demonstrated in this experiment.

Project Description:

Part I.

F. The Effect of Prolonged Anticoagulation on Spontaneous Tumor Metastases

Objectives:

1. To evaluate the effect of long-term anticoagulation on the size of a primary tumor and its spontaneous metastases in an isologous host system.

2. To observe morphology and fibrin content of a tumor growing in an anticoagulated animal. The coagulation mechanism is believed to play a role in the initial adherence of embolic tumor cells and subsequent development of distant metastases. Transient anticoagulation decreases the number of pulmonary metastases in animals give tumor cells intravenously. This study evaluates the effect of long-term anticoagulation on the size of a primary tumor and its spontaneous metastases in an isologous system.

Methods Employed:

Two host-tumor systems were studied: The methylcholanthrene sarcoma T241 of Lewis in C57BL/6N mice and the spontaneous mammary adenocarcinoma in C3H/HeN mice. Following tumor inoculation one-half of the mice in each system were begun on oral sodium warfarin at a constant pre-determined dosage. At five intervals equal numbers of control and anticoagulated mice were sacrificed after prothrombin time determinations. By India ink differential staining of tumor and lung tissue, metastases were detected macroscopically.

Major Findings:

Prothrombin times of the mice were maintained continuously in the range of 2 to 3 times normal and the mice free of any hemorrhagic complications. In each of the five intervals of comparison in both host-tumor systems, at least three times as many controls as anticoagulated mice had metastases. For the C57BL/6N mice, a cumulative total of 73% of controls and 15% of anticoagulated mice had metastases and for the C3H system, 62% of controls and 15% of these anticoagulated.

Significance to Biomedical Research and Program of the Institute:

The results support the earlier work done with an early generation of methylcholanthrene induced fibrosarcoma. In all three systems long-term anticoagulation has significantly reduced the incidence of spontaneous metastases and significantly increased the number of individuals free of metastatic disease. The more recent studies show the effect of anticoagulation will persist when the level of anticoagulation is maintained constantly in a safe range free of any hemorrhagic complications. This further attests to the importance of the coagulation mechanism in the intravascular metastatic spread of tumors.

Proposed Course:

Investigation of the effect of anticoagulation in the I.V. dissemination of tumor cells now in progress are designed to disclose the level and duration of anticoagulation both before, during, and after the dissemination of tumor cells that is necessary to prevent their implantation and colonization.

Project Description:

Part II:

A. The Stimulation of Dormant Cancer Cell Growth by Hepatic Trauma

Objectives:

To provide a model for the study of dormant cancer cells.

Methods Employed:

A critical aspect of the problem of cancer metastases revolves about the dormant cancer cell. Clinically it has been repeatedly documented that patients many years after resection of a primary lesion and apparent cures suddenly succumb to widely disseminated and rampantly progressive metastatic growth after minor trauma or major surgery. An experimental model for dormant cancer cells was reported by Bernard Fisher in Pittsburgh in 1959. Two weeks after injection of Walker 256 cancer cells into the portal vein of rats, liver massage produced gross metastases which otherwise would not develop. A study of dormant cancer cells was undertaken using this same model. The first step listed below was to demonstrate this phenomenon repeatedly and verify these experimental results. A cytosieve preparation of solid Walker 256 tumor was prepared under sterile conditions. Five thousand, 25,000, and 125,000 viable tumor cells as proven by trypan blue exclusion were injected into the portal vein of 150gm. Sprague-Dawley rats at laparotomy. The abdomen was closed with interrupted through and through sutures of silk. At 4, 6, and 8 weeks of post-injection athe animals were opened and livers grossly negative for metastases at that time were manipulated. All surrounding attachments of liver were taken down, the liver compressed, and in essence a sham-hepatectomy performed. These animals were again closed. At 4 week intervals thereafter the animals were re-massaged until tumor developed or until 8 massages had transpired. The number of animals in control groups having metastases and the number of dormant cells stimulated to clinical growth were tabulated. Under the same protocol used in the study of the Walker 256 tumor in Sprague-Dawley rats, a 3-4 benzpyrene induced sarcoma in syngeneic Fisher rats was studied. Preliminary studies showed that this tumor was highly immunogenic in this rat strain. Contrasting results with this animal tumor system compared with that obtained with the weakly immunogenic Walker 256 tumor may show the effect of tumor immunity on the spread of dormant tumor cells.

Major Findings:

With the 5,000 cell injection of Walker 256 tumor no animals developed metastases. With repeated massage 64% of 3 series (40 to 45 animals per series) developed static metastases - biopsy proven. With the 25,000 and 125,000 cell control animals about 10% developed metastases in each group. With serial passage a total of 70% developed hepatic metastases. The attempt to demonstrate dormant cells in syngenic Fisher rats using a 3-4 benz(a(pyrene induced sarcoma was not successful. Ninety-eight animals receiving subthreshold intraportal doses of this tumor serially underwent 6 liver massages at 4-week intervals. In no instance were "dormant cells" stimulated to gross or microscopically discernible growth.

Significance to Biomedical Research and the Program of the Institute:

The 3-4 benzpyrene sarcoma was shown to be highly immunogenic in Fisher rats. The Walker 256 tumor was not demonstrated immunogic in Sprague-Dawley rats. It is, therefore, hypothesized that the ability of a tumor to establish itself

in the dormant state might be related to the immunogenicity of the tumor itself. The highly immunogenic tumor cells were destroyed by host defense of overcame them. Weakly immunogenic tumors could apparently co-exist with host defenses in a non-driving state until recalled by surgical stress. A study which potentially could delineate factors governing the resurgent growth of dormant cancer cells would be of utmost importance in the control of clinical disease. Such late recurrences occurring 2 to 15 years postoperatively account for a major proportion of surgical failures.

Proposed Course:

Continued study of the dormant tumor cell state with this experimental model should provide insight into the mechanism underlying the phenomenon of late tumor recurrence in human cancer patients.

Project Description:

Part II.

B. Differences in Immunogenicity Between Primary Tumors and Their Metastases

Objectives:

To test the hypothesis that antigenic differences exist between various metastases from a primary tumor and between these metastases and the primary tumor.

Methods Employed:

A fibrosarcoma was induced in sygeneic Fisher rats by injection of benzpyrene suspended in mineral oil. Immunogenicity was demonstrated in the third and fourth generations of the tumor. One rat was chosen for study, which had three large discrete metastases in the lungs. They were dissected free and designated respectively as M0, M1, and M2. Differences in the immunogenicity between these metastases and the primary tumor, designated P, were determined by the classical methods of tumor inoculation, amputation, and tumor challenge.

Major Findings:

M0 produced tumor in 27/30 controls versus 0/10 M0-immunized rats ($P<.01$). M2 produced tumors in 11/30 controls versus 0/10 M2-immunized rats($P<.01$). P grew 21/30 controls versus 0/10 P-immunized rats ($P<.01$). These data show that M0, M2, and P lines are all significantly immunogenic. However, there was no significant inhibition of M0 or M2 tumor incidence in P-immunized rats, growing in 11/10 and 9/12, respectively, and similar to their growth in 27/30 and 11/30 controls, respectively. Mean latency periods of M0 and M2 in P-immunized rats were similar to those in control rats. These data indicate that the cell suspension derived from transplanted metastases grew unhibited in rats immunized with the primary. Additional comparisons are of interest. M0 or M2

immunization significantly decreased the take of P compared to its growth in controls-1/9 in MO immunized and 4/11 in M2-immunized versus P growth in 21/30 controls ($P<.05$). Immunization with MO did not significantly inhibit the take of M2 (5/10) compared to growth in 11/30 controls and immunization with M2 did not significantly decrease the take of MO (7/10) compared to growth in 27/30 controls. However, tumor latency periods were prolonged. These latter comparisons indicate that the metastases can be immunogenically dissimilar not only from the primary but also from each other and that some cross-reactivity may persist.

Significance to Biomedical Research and Program of the Institute:

These data offer considerable support for the concept that metastases may develop as a consequence of differential immunogenicity between the primary tumor and its metastases. Reasoning thus, tumor hosts may be unable to reject cells shed from the primary tumor because they are immunologically different from the primary. The consequences of this for eventual clinical application of immunotherapy are great in that most immunotherapy will be directed against metastatic disease. Hence, the differential immunogenicity of the metastatic tumor may have to be taken into account in developing modalities for immunotherapy.

Proposed Course:

This experimental model will be adopted for the study of differences in immunogenicity between primary tumors and metastases in human cancer patients.

Project Description:

Part II.

C. Effect of Tumor Immunogenicity on the Metastatic Potential of Circulating Tumor Cells from Tumor Metastases

Objectives:

To determine the effect of tumor-specific immunity on the propensity for tumor formation by circulating tumor cells shared from metastatic tumors.

Methods Employed:

Tumor systems were used: An immunogenic MCA-induced tumor and a non-immunogenic S91 melanoma. C57BL/6N mice were used with the first tumor and CDF1 mice with the second. The design was identical in both experiments. Mice with previously amputated primary tumor bearing legs and pulmonary metastases were killed and their left heart blood aspirated. The blood was used to inoculate mice pairs intravenously (I.V.). These recipients were designated Group 2 mice. The pairs were divided into 2 groups, Group 2A and Group 2B. Each of these groups were further divided into two additional groups according to the time of sacrifice after inoculation of blood: Group 2A-1 was killed 24 hours after inoculation; Group 2B-1 was killed one week after inoculation, and Group

2A-2 and 2B-2 were killed six weeks after inoculation. When Group 2 mice were killed, left heart blood was aspirated and inoculated into normal recipients designated Group 3. These mice received the same subgroup designations as the Group 2 blood donors. When several mice in Group 3A-2 and 3B-2 died approximately six weeks after inoculation, all Group 3 mice were killed.

Major Findings:

The results were similar with both animal tumor systems studied. In the C56Bl/6N mice inoculated with MCA-induced tumors, no lung tumors were found in Group 2 mice that were killed 1 day and 1 week after I.V. inoculation of blood containing circulating tumor cells, nor were any found in Group 3 mice inoculated with blood aspirated from these Group 2 mice. In Group 2 animals killed six weeks after inoculation, a high incidence of lung tumors were produced: 14/18 in Group 2A and 13/16 in Group 2B-2. Also, lung tumors were produced in Group 3 animals inoculated with blood from these mice: 5/10 in Group 3A-2 and 5/11 in Group 3B-2. In the CDF1 mice inoculated with S91 melanoma, Group 2 animals killed on one day and one week after tumor inoculation had no lung tumors, neither did their respective Group 3 blood recipients. On the other hand, a total of 35/42 Group 2 animals had lung tumors when killed six weeks after inoculation and the blood from these animals produced lung tumors in 15/30 in Group 3 recipients. These results showed that blood from mice with pulmonary metastatic tumors readily produced lung tumors when inoculated into normal mice. In Group 2 mice the possibility existed that the lung tumors produced may have generated from cells shed from the previously amputated primary tumor. However, the subsequent transfer of blood to other mice (Group 3) produced tumors only with blood from Group 2 mice that had large lung tumors, thus demonstrating that their origin is in circulating tumor cells shed from pulmonary tumors, not from the primary tumor. The experiment thus demonstrates that pulmonary metastases may shed cells that survive and produce new tumors and since the results were the same with an immunogenic tumor as with an non-immunogenic tumor. It demonstrates that cells from metastases of an immunogenic tumor enjoy the same facilitation in their propagation as cells from non-immunogenic tumors.

Significance to Biomedical Research and Program of the Institute:

These observations may have clinical significance to one of the most perplexing problems in clinical oncology the metastatic potential of tumor metastases. Frequently, in a patient with a controlled primary tumor and a single metastases deposit, a waiting period is elected to allow for the appearance of additional metastases. This course entails a theoretical danger of allowing the dissemination of cells from the metastases that produce tumors elsewhere. The data gives no support to the hypothesis often advanced that immunity may destroy circulating tumor cells. Until data from human experiments show that this phenomenon does not occur in humans, the clinician must consider the experimental evidence which indicates that metastatic deposits shed cells that can colonize elsewhere in the tumor-immunized host.

Proposed Course:

Publication of Data

Project Description:

Part II:

D. Immunity of the Autogenous Host to Viable Tumor Cells Shed from Pulmonary Metastases

Objectives:

The phenomenon of preferential metastases as vividly demonstrated by Kinsey was used to explore the autogenous biologic potential of viable tumor cells shed from pulmonary metastases. This study has direct bearing on the question, "Do metastases metastasize?," and also has major implications for the immunity of the host cells circulating in the blood stream.

Methods Employed:

First, preferential metastasis was demonstrated in the fifth generation of a methylcholanthrene-induced sarcoma of C57 mice in syngeneic C57 black mice. Heterotopically transplanted organ fragments of newborn syngeneic C57 mouse lung, kidney, and liver were aseptically placed subcutaneously. Forty-eight hours before transplantation of these organs the animals received a transplant of 10^6 tumor cells in the leg. Spontaneous metastases occurred only to pulmonary tissue, whether it be the animals own tissue or the transplant. Other organs and other transplants were never developed tumor. In a second group of experiments, transplants were placed after amputation of a large leg primary, amputating having been performed at a stage where pulmonary metastases develop in the pulmonary transplant, and all the pulmonary metastases in the host were of approximately the same size. These data suggest that pulmonary metastases themselves are not capable of remetastasizing to sites of preferential metastases. In a group of untreated animals, the left heart blood was removed and inoculated into syngeneic recipients. In eight of eight such bioassy recipients, tumor growth occurred, demonstrating the presence of viable circulating tumor cells shed from the pulmonary metastases in left heart blood.

Significance to Biomedical Research and Program of the Institute:

It was concluded that within the context of this tumor-host system, which is a highly immunogenic tumor-host system, that metastases do not metastasize. Limitations on the interpretation of the data are imposed by the nature of the preferential sites, since they are tumor implants of organs into muscle. Nevertheless, the findings warrant further exploration of this unique model for elucidation of functions related to sites of preferential metastases in human cancer patients.

Proposed Course:

Study of the phenomenon of preferential metastases should provide insite into factors that determine the colonization of circulating tumor cells. This model appears to provide of reproducing the phenomenon and therefore will be used to determine local factors that favor or inhibit the growth of metastatic tumor cells.

Objective and Approach of the National Cancer Program:

Objective 1: To reduce the effectiveness of external agents in increasing the probabilities of development of cancers in existing individuals or in individuals of subsequent generations.

Approach 4: Improvement of animal models of human cancer and evaluation of the effect of combined factors.

Publications:

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Serial No. NCI-3806
1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Surgical Physiology and Metabolic Changes Associated with Extensive Cancer Surgery

Previous Serial Number: Same

Principal Investigator: Robert C. Hoye, M.D.

Other Investigators: A.S. Ketcham, M.D., S.H. Bennett, M.D., G.W. Geelhoed, M.D., G.C. Riggle, B.S., and R. Arron, M.D.

Cooperating Units: Department of Anesthesiology, Blood Bank, Department of Nursing, Radiopharmaceutical Service, and Biomedical Engineering and Instrumentation Branch, Clinical Center, NIH

Man Years:

Total:	4
Professional:	2
Other:	2

Project Description:

Part I: Albumin Kinetics with Trauma and Hemorrhage in Man and Animals

Part II: Capillary Permeability Studies in Man and Animals

Part III: Studies on the Etiology, Pathogenesis, and Treatment of the Syndrome Post-Traumatic Pulmonary Insufficiency

Part IV: Feasibility Studies on the use of Auto-transfusion Methods in Major Cancer Surgery

Project Description:

Part I: Albumin Kinetics with Trauma and Hemorrhage in Man and Animals

Objectives:

Previous studies in this laboratory have indicated that after major cancer surgery a marked disproportionate plasma volume deficit occurs amounting to 24% of the patient's plasma volume immediately prior to surgery. In addition, a 33% deficit of the total circulating protein has been noted in patients immediately following major operative procedures. These studies are designed to quantitate the extent of albumin depletion in man and animal with operative trauma and to determine, if possible, the magnitude and direction of the shifts involved.

Methods Employed:

The plasma volume total exchangeable albumin, total circulating albumin, albumin distribution ration, and fractional catabolic rate are being studied with conventional isotopic methods. When indicated patients are studied for 7-10 days at each measure interval and all losses measured at surgery and, in selected patients, whole body counting has also been employed. In measurement of the total circulating albumin in patients or animals, direct measurement of the plasma volume is determined at each measurement interval as well as determination of albumin by serum electrophoratic methods.

Major Findings:

In a series of nine patients undergoing regional node dissection, we have found that the total body albumin pool is decreased by 29% of the preoperative measurement during the course of surgery. This amounted to 80 grams of albumin and only a small portion of this loss could be accounted for by external measurements. Whole body counting indicated that this albumin loss from the exchangeable body pool was sequestered in an uncirculating albumin pool which demonstrated a decrease of 27% over the time of the operative procedure. In control patients no changes in the albumin pool were noted. In view of the findings in these patients who underwent the regional node dissection it is anticipated that with operative procedures of two to three times the magnitude of a single regional node dissection it can be expected that between 160 and 240 grams of albumin will be sequestered.

In the 12 primates studied, it was found that intestinal manipulation caused a sequestration of albumin in the intestinal tract which amounted to 24% of the total circulating albumin pool present prior to the operation. In nine dogs as studied, it was observed that a simple non-shocking hemorrhage of 30% of the measured total blood volume resulted in a continued additional loss of albumin from the intravascular compartment in the six hours following the hemorrhage which amounted to 33% of the total circulating albumin present in the circulation at the end of the hemorrhage period.

Other investigators had previously indicated that following hemorrhage in normal volunteers there was a rapid influx of albumin into the vascular compartment. Based on previous work in this laboratory this was not felt to be the situation in the postoperative patient and 13 patients were investigated. The total circulating albumin pool was measured before surgery. In the eight patients of the group who underwent major intra-abdominal resectional surgery the immediate postoperative total circulating albumin pool was 72% of the amount present in the preoperative period. This indicated net loss from the total circulating albumin pool of 28%. By six hours after surgery this deficit was 22% and at 12 hours after surgery this deficit was 19%. These studies indicated that following operative trauma and albumin sequestration there was not a rapid influx of albumin into the vascular compartment as had been suggested by other investigators following studies on normal volunteers but that instead there was a very slow influx which by 12 hours after the operative procedure had replenished only one-third of the deficit which had occurred during the surgical procedure.

Significance to Biomedical Research and Program of the Institute:

These studies in animals and man indicate rather conclusively that with major surgery and trauma there is a sequestration of a significant portion of the total body albumin pool and that this amount of albumin remains sequestered for a considerable length of time. If normal homeostasis is to be maintained following the surgical procedure then large amounts of albumin infusions should be administered both during and after the operative procedure. If albumin is not administered the speed of plasma volume re-expansion after hemorrhage will be slowed and, in addition, there will be a lowering of the colloid osmotic pressure which may be responsible for the interstitial pulmonary edema occurring near the end of surgery or in the immediate post-operative period in some patients. Based on previous work it is felt that one of the major reasons for this loss of total circulating albumin is secondary to a 75% decrease in the amount of albumin being returned to the intravascular compartment via the thoracic duct lymph during the surgical procedure. In previous studies the thoracic duct has been cannulated and the flow rate and protein content of lymph being returned to the vascular system measured in patients undergoing surgery.

Metabolic changes following extensive cancer surgery have not previously been thoroughly investigated. To date the quantitative studies being carried out by this laboratory in patients undergoing extensive cancer surgery have indicated that a multiplicity of deficits may occur. With the increased knowledge obtained in these studies the management of the postoperative patient has changed dramatically in recent years. The morbidity and mortality has decreased significantly with the institution of new treatment regimens.

Proposed Course:

Future studies will continue to investigate these parameters in selected patients and animals while manipulating the type of replacement fluids given

during the period of surgical stress.

Project Description:

Part II: Capillary Permeability Studies in Man and Animals

Objectives:

Patients who undergo extensive surgery in the treatment of cancer have been demonstrated to develop a disproportionate plasma volume deficit, a functional extracellular fluid volume loss, and a decrease in the total circulating albumin pool. To maintain adequate circulation these patients must be given large amounts of plasma, albumin, fluids, and electrolytes in the immediate postoperative period. There are reasons to believe that this phenomenon may be, at least in part, due to a generalized increase in capillary permeability.

Investigations in this area of study to date have been done by following the disappearance from the blood stream of radioactive labels. Such labels, at 60,000 molecular weight, may have been too large to identify significant changes in capillary permeability. A new method has been developed to measure capillary permeability over a wide molecular weight range and it is proposed to use this method in studying changes in capillary permeability with trauma and hemorrhage.

Methods Employed:

By using gel columns composed of sepharose, dextran can be fractionated into its various molecular weight components. It has previously been demonstrated using this method that the permeability of the capillary membrane to molecules from 10,000 to 200,000 can be determined with accuracy and reliability. The capillary permeability which occurs in response to hemorrhage, shock, and operative trauma will be followed in animals by following the disappearance from the blood stream of dextran molecules in molecular weight range of 10,000 to 100,000. Studies will be performed before and after the traumatic episode and the disappearance rates of the various molecular weight components of dextran administered will be compared. To facilitate interpretation of the results the renal excretion of the dextran molecules will be prevented by previous bilateral nephrectomy.

The permeability of various vascular beds will be studied in subsequent experiments as follows. At the time of nephrectomy the lymph channels draining the liver will be clamped, cut, and ligated at their point of entrance into the cisterna chyli. At the time of study, samples of the lymph from the thoracic duct and blood from the celiac axis and inferior mesenteric artery will be collected. After the fractionation of the dextran into its various molecular weight components the sieving characteristics of the intestinal circulation following a traumatic episode can be determined. In a similar manner, the sieving characteristics of the hepatic bed can be determined with prior ligation of the intestinal lymphatics to the cisterna chyli. The

sieving characteristics of the pulmonary capillary bed will be studied by obtaining right thoracic duct lymph which drains the major portion of the pulmonary bed. Simultaneous with these studies measurements will be made of the plasma volume, extracellular fluid volume, total circulating albumin, serum colloid osmotic pressure, blood p^H, and blood gases.

Depending upon the changes observed in these studies, attempts will be made to study the response of the general capillary bed to drugs and various fluid regimens administered at the time of the surgical trauma.

The experimental animals used in these studies will be those found to be most suitable for analyzing permeability characteristics of the capillary bed under investigation. For the intestinal and hepatic capillary beds it is felt the primate will be most suitable and for the study of the pulmonary capillary bed the larger calf or goat is anticipated to be a more suitable experimental animal.

Major Findings:

Using this method it has been demonstrated that after a period of hemorrhagic shock in splenectomized dogs that a slight increase in capillary permeability for molecular sizes below 40,000 occurred during and after the hemorrhagic shock phase. This occurred despite maintenance of a normal arterial tension.

The method has also demonstrated that after localized radiation injury there is a marked increase in capillary permeability for the fraction from 10,000 to 30,000 molecular weight. With the passage of time during the first two weeks after the radiation injury the amount of capillary permeability occurring increased.

Significance to Biomedical Research and Program of the Institute:

The immediate significance of this work is improvement of the pre- and post-operative management of patients undergoing major cancer surgery.

Proposed Course:

The major portion of this year has been used in a standardization of gel columns by using dextran fractions of normal molecular weight. In addition automatic methods for the determination of dextran have been developed on the autoanalyzer and are currently being programmed into a computer. It is anticipated that in the coming year significant results will be obtained to answer a few of the many questions concerning homeostasis which arised during the course of performing extensive cancer operations.

Project Description:

Part III: Studies on the Etiology, Pathogenesis, and Treatment of the Syndrome Post-traumatic Pulmonary Insufficiency

Objectives:

It is estimated that among adult patients who die in intensive care facilities following severe injuries 30 to 50% will die from progressive pulmonary failure. Many factors have been implicated in this syndrome of post-traumatic pulmonary insufficiency. Underlying essentially all cases, however, is a low flow state precipitated by injury, operation, hemorrhage, or sepsis. In the resuscitation of the patient with this condition, massive homologous blood transfusion is typical. While this therapy has unquestioned indication for the restoration of circulatory homeostasis, there is considerable evidence that the transfusion of multiple units of aged bank blood may be deleterious to the lungs due to the cellular debris infused with blood. To study the effects of infusion of stored blood on the lungs we have devised an experimental model using an in vivo pulmonary lobe perfusion system in both the dog and the baboon.

Methods Employed:

Autologous blood was stored for 21 days in ACD solution, passed through a standard filter, and perfused for 2 hours through the left lower lobe of 7 dogs and 7 baboons. Seven animals of each species served as controls and were perfused with autologous filtered blood stored for 24 hours. The blood was passed through a membrane filter for deoxygenation and into the pulmonary artery at constant temperature and flow rate. Pulmonary arterial (P_{pa}) perfusion pressures and gas tensions (by mass spectrometry) were continuously monitored while pulmonary venous pressure was maintained at zero. The lobe was ventilated with room air via a Carlens tube and a volume respirator, and end-inspiratory bronchial pressures (P_b) were recorded continuously. Pressure flow curves through a range of 100 to 600 ml/min were obtained at 30 minute intervals and lung biopsies were taken before and at the conclusion of the perfusion.

Major Findings:

In the dog, the mean increase in P_{pa} was 103% over controls and the P_b increased 58%. Arteriovenous (A-V) oxygen gradients decreased 64% compared to controls. Wet/dry lung weight ratios increased by a mean of 17% in the experimental group relative to controls and gross and microscopic evidence of pulmonary edema was seen. In the baboon, the mean increase in P_{pa} was 125% and the mean increase in P_b was 100% over controls. Wet/dry lung weight ratios increased by a mean of 16% over controls. Six of 7 baboons demonstrated frothy fluid in the airway, and gross and microscopic examination confirmed the presence of pulmonary edema. These results were all significant at $P<.05$. The mean A-V pO_2 difference was 43 (range = 18-100%) compared with the control mean of 17% (range = 3-27%). The pCO_2 differences remained constant in both species.

Significance to Biomedical Research and Program of the Institute:

Post-traumatic pulmonary insufficiency or shock lung has been recognized in recent years to be one of the main causes of death in patients following extensive surgery or trauma. The etiology of this syndrome is obscure but probably results from several contributing factors. The relative importance of massive homologous blood transfusion is not entirely clear, but the potential damage to lung parenchyma by the infusion of aged blood has been suggested in our experimental model. As our model becomes further developed we anticipate that it will be possible to identify the substances in blood which are producing lung damage, and once having established the pathogenesis will be able to intelligently manage the seriously ill patient in whom massive transfusion is indicated.

Proposed Course:

In the coming year we anticipate further study on the effects of stored blood on the lung parenchyma. We intend to alter the blood in such a way that we may be able to identify the elements in blood which are most responsible for the pulmonary injury. This will include filtration, perfusion with cells or serum which has been aged and mixed with cells or serum from a fresh unit of blood, and the use of pharmacologic agents which may help in ameliorating the pulmonary injury.

Project Description:

Part IV. Feasibility Studies on the Use of Auto-transfusion Methods in Major Cancer Surgery

Objectives:

Auto-transfusion has been used with success over the past 80 years largely in emergency situations to restore blood volume following acute blood loss when no donor blood has been available. It has also been used successfully in a limited number of elective surgical cases including several cases of oncologic surgery. This surgical area appears to be particularly applicable to auto-transfusion because of the significant blood loss encountered.

The purpose of this study is threefold: (1) to determine whether auto-transfusion is a safe and effective means of blood replacement in a patient undergoing extensive cancer surgery; (2) to examine specific effects of auto-transfusion on blood cellular elements, and (3) to develop a method of anti-coagulating blood suctioned from the operative wound without employing systemic anticoagulation.

Major Findings:

To date all studies have been done in animals in preparation for clinical trials in cancer patients undergoing major surgery.

We have examined the effects of auto-transfused blood on renal and pulmonary function. To ascertain the effects of auto-transfused and consequently hemolyzed blood on renal function, 10 dogs were used as subjects in the investigation. An estimated 25% blood volume was removed from each dog, lysed with distilled water and reinfused. The animals were observed to develop hematuria, acutely, which gradually cleared. Serial creatinine and BUN determinations were done and found to be normal over four postoperative days. In this group of animals we observed no significant change in respiratory rate or depth of respiration which might indicate pulmonary insult. This response was typical of more than 40 dogs in whom auto-transfusion has been carried out. It is significant to note, however, that screen filtration determinations were elevated in animals which were auto-transfused with blood exposed to the peritoneal cavity. This elevation suggests that microaggregates are present in the auto-transfused blood but may be so small or so unstable as to cause no significant injury to the lung.

Significance to Biomedical Research and Program of the Institute:

A suction device developed by the Bio-engineering Division to our specifications has been modified and improved such that it is a reliable tool for the anticoagulation of auto-transfused blood. Reversal of heparin effects in the animal has been accomplished by "in line" protamine infusion through a double lumen intravenous catheter tip. Clotting studies indicate that this system is capable of maintaining complete anticoagulation in the extracorporeal circuit and normal clotting times in the animal. At present an active investigation is being carried on to refine current methods for monitoring of heparin anticoagulation in the patient. Specifically, 2 methods are being evaluated which appear to be more reliable and much easier to administer than the Lee White clotting time.

Proposed Course of Project:

With the refinement of the anticoagulation system and the acquisition of disposable filtering devices already used clinically for auto-transfusion we anticipate clinical trials in the near future.

Objective and Approach of the National Cancer Program:

Objective 6: To cure as many patients as possible and to maintain maximum control of the cancerous process in patients not cured.

Approach 5: To develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (alone) and in combinations) in the cure and palliation of cancer.

Publications:

Hoye, R.C., Voightlander, V., Plantin, L.O., and Birke, G.: Changes in the total circulating albumin, plasma volume and extracellular fluid volume with hemorrhage and the response to hydrocortisone. Acta. Chir. Scand. 137: 299-304, 1971.

Bennett, S.H., Hoye, R.C., and Riggle, G.C.: Intraoperative autotransfusion - preliminary report of a new blood suction device for anticoagulation of autologous blood. Amer. J. Surg. 123: 257-260, 1972.

In press:

Hoye, R.C., Bennett, S.H., Geelhoed, G.W., and Gorschboth, C.: Fluid volume and albumin kinetics occurring with major surgery. J.A.M.A.

Serial No. NCI-3816
1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NCI
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cryobiology

Previsou Serial Number: Same

Principal Investigators: A.S. Ketcham, M.D., W.G. Hammond, M.D., and R.M. Beazley, M.D.

Other Investigators: H.B. Neel, III, M.D., L.B. Thomas, M.D., Mr. Grant Riggle, Mrs. R.B. Hume

Cooperating Units: Laboratory of Pathology, NCI, and Biomedical Engineering and Instrumentation Branch, NIH

Man Years:

Total:	1-1/2
Professional:	1/2
Others:	1

Project Description:

Part I. Apparatus Modifications for Experimental Cryogenic Surgery of Cancer

Part II. Potentiation of Cryonecrosis by Inflow Occlusion and Repetitive Freezing Cycles

Part III. Cryosurgical Studies on a Functional Non-endocrine Tumor

Project Description:

Part I. Apparatus Modifications for Experimental Cryogenic Surgery of Cancer

Objectives:

To develop an apparatus which will enable the investigator to surgically perform a standardized, controllable cryogenic lesion during cryosurgery of cancerous tissue.

Methods Employed:

The Linde CE2, PR-5 probe was modified so it could be mounted in a microscope stand from which the barrel lens system had been removed. High speed micro-miniature-thermocouples were attached to the cryoprobe by means of a Teflon holder so that tissue temperatures 2 mm. and 5 mm. from the cryolesion could be monitored as the cryolesion developed.

Major Findings:

It was determined that visual monitoring of the cryolesion was unsatisfactory for treating cancer. Proper cryodestruction can only be accomplished by tissue temperature monitoring. The above described apparatus was quite suitable for experimental animal use.

Significance to Biomedical Research and Program of the Institute:

This apparatus modification and data gleaned from its experimental use will allow more standardized application of cryolesion. This will have significance not only in the primary treatment of tumors but also in the development of immunological models to study the effect of cryodestruction on immunological mechanisms of the organism. Furthermore, it is felt that this modification of existing methodology will be an advancement in surgery and in immunology which will help in cure or palliation of cancer patients.

Proposed Course:

Publication of data. These experimental studies will continue with the plan to eventually use them in man for tumor destruction.

Project Description:

Part II. Potentiation of Cryonecrosis by Inflow Occlusion and Repetitive Freezing Cycles

Objectives:

To observe the pathophysiological consequences of repetitive freezing and the effect of total inflow occlusion on the extent of cryonecrosis.

Methods Employed:

Primate liver tissue was frozen in situ repetitively with and without inflow occlusion. Extent of tissue necrosis was observed by pathological sectioning of the treated organ.

Major Findings:

(1) This freezing method was well tolerated by the animal. (2) There were no serious side effects secondary to cryosurgery of primate liver tissue. (3) Repetitive freezing and slow thawing increased the size of cryodestruction. Inflow occlusion appeared to potentiate freezing and increase thawing time, thus ultimately increasing cryodestruction.

Significance to Biomedical Research and Program of the Institute:

This project clearly demonstrated that cryosurgical lesions could be placed in primate liver with safety. Additionally, it showed that repetitive freezing and inflow occlusion would potentiate cryonecrosis. This may have direct application to treatment of hepatomas or large irresectable tumors in surgical patients. Furthermore, it has consequence in the development and utilization of new methodologies in the surgical approach to cure and palliation of cancer patients.

Proposed Course:

Publication of data. These experimental studies will continue with the plan to eventually use them in man for tumor destruction.

Project Description:

Part III. Cryosurgical Studies on a Functional Non-endocrine Tumor

Objectives:

To ascertain some of the parameters involved in cryosurgery of an experimental prostatic cancer. Also to assess the relationship between tumor destruction and the change in serum acid phosphatase levels.

Methods Employed:

Hamster prostatic carcinoma induced in vitro by SV-40 virus transformation was injected in cell suspension into the flank of hamsters. These tumors were treated cryosurgically and acid phosphatase levels were monitored.

Major Findings:

It was noted that the acid phosphatase level was detectable prior to the time the tumor becomes palpable. Following cryosurgical treatment if a cure has been instituted serum acid phosphatase falls to normal levels and remains there. If cure has not been gained then acid phosphatase returns to normal and then transiently begins to rise again. This tumor system was also studied in an attempt to demonstrate differential immunity. Immune animals failed to develop palpable tumors from subsequent challenge but did develop a transient rise in serum acid phosphatase following injection of the challenge dose of tumor.

Significance to Biomedical Research and Program of the Institute:

This promises to be a useful model for studying the effects of cryosurgery on prostatic tumor and also the biochemistry of serum acid phosphatase values in monitoring such a tumor. It is a further development in the methodology of surgery in an effort to cure and palliate cancer patients.

Proposed Course:

Publication of data. These experimental studies will continue with the plan to eventually use them in man for tumor destruction.

Objective and Approach of the National Cancer Program:

Objective 6: To cure as many patients as possible and to maintain maximum control of the cancerous process in patients not cured.

Approach 1: Develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (alone) and in combinations) in the cure and palliation of cancer.

Publications:

Neel, H.B., III, Riggle, G.C., Myers, R.S., Ketcham, A.S., and Hammond, W.G.: Apparatus modifications for experimental cryogenic surgery of cancer. J. Cryobiology 8: 501-505, 1971.

Neel, H.B., Ketcham, A.S., Hammond, W.G.: Ischemia potentiating cryosurgery of primate liver. Annals of Surgery 174: 309-318, 1971.

Neel, H.B., Ketcham, A.S., Hammond, W.G.: Cryonecrosis of normal and tumor bearing rat liver potentiated by inflow occlusion. Cancer 28: 1211-1218, 1971.

Neel, H.B., Hammond, W.G., Ketcham, A.S.: Cryosurgery of SV40 prostatic transplant tumors. International Surgery 57: 61-66, 1972.

Ketcham, A.S., Hammond, W.G., Myers, R.S., Neel, H.B., III: Experimental cryosurgery. Panminerva Medica 13: 468-472, 1971.

Serial No. NCI-3817
1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Subcellular Mediation of Immune Responses to Tumor Antigens

Previous Serial Number: Same

Principal Investigator: A.S. Ketcham, M.D., Y.H. Pilch, M.D.

Other Investigators: P.J. Deckers, M.D., B.R. MacPherson, M.D., K.P. Ramming, M.D., and R.B. Hume

Cooperating Units: None

Man Years:

Total:	3-1/2
Professional:	1-1/2
Other:	2

Project Description:

Part I. Transfer of Tumor Specific Immunity with RNA: Inhibition of Growth of Isografts in Syngeneic Animals

Objectives:

To isolate preparations rich in ribonucleic acid from cells and tissues which process antigens, such as lymph nodes and spleens of the animals immunized with tumors and to study the immunoreactive properties of these RNA preparations in mediating immune responses to tumor antigens. To utilize these preparations for immunotherapeutic purposes in experimental animal models.

Methods Employed:

RNA is prepared by phenol extraction of lymph nodes and spleens or peritoneal macrophages of experimental animals following immunization in vivo or in vitro with tumor cells or following immunization of animals by temporary tumor growth. RNA is determined by the orcinol reaction, DNA by the diphenylamine reaction, and protein by the Lowery method. RNA is characterized by its sedimentation characteristics upon ultracentrifugation on sucrose density gradients. The tumor-host systems employed have been benzpyrene-induced sarcomas in inbred mice, methylcholanthrene-induced sarcomas in inbred mice, benzpyrene-induced sarcomas in inbred rats, and methylcholanthrene-induced sarcomas in inbred mice, benzpyrene-induced sarcomas in inbred rats, and methylcholanthrene-induced

sarcomas in inbred guinea pigs. Cellular immune responses were determined in vitro by a modification of the immune cytolysis assay of McKhann and in vivo by the ability of the immunized animal to demonstrate a significant reduction in the incidence of tumor development.

Major Findings:

RNA was extracted from the lymph nodes and spleens of guinea pigs previously immunized with benzpyrene-induced C_3HF-HeN mouse sarcoma (BP4). Normal C_3H spleen cells were incubated with this RNA and injected into C_3H mice which were then challenged with 10^4 viable BP4 cells. Marked decrease in the incidence of tumor growth resulted. RNA from guinea pigs immunized with normal C_3H tissue was ineffective. Treatment of the active RNA preparations with ribonuclease removed activity while deoxyribonuclease or pronase treatment did not. Specificity of this response for the immunizing tumor was demonstrated. In another system, RNA from the nodes and spleens of guinea pigs immunized with the Fisher 344 rat sarcoma (BP-1R) was mixed with dextran sulfate, a potent inhibitor of rebonuclease, and administered to normal Fisher rats, which were then challenged with 10^4 BP-1R cells. Significant reduction in the incidence of tumor development was noted. Dextran sulfate alone or with nonspecific guinea pig RNA was ineffective.

In a syngeneic system designed to conclusively establish RNA mediation of immunity to tumor-specific transplantation antigens, normal Fisher spleen cells were incubated with RNA from the spleens of Fisher rats immunized with BP-1R and given IV to Fisher rats which then received 3.5×10^4 BP-1R cells. Inhibition of tumor growth resulted. In another syngeneic system, in vitro, normal strain 2 guinea pig spleen cells were incubated with RNA extracted from the spleens of strain 2 guinea pigs immunized with a methylcholanthrene-induced strain 2 guinea pig sarcoma (MCA-A). Acellular areas of immune cytolysis of MCA-A cells were produced when RNA-incubated spleen cells were applied to MCA-A monolayers, but produced no cytolysis of normal strain 2 lung epithelial cells. Again, ribronuclease treatment abrogated the response, and specificity for the immunizing tumor was demonstrated.

Significance to Biomedical Research and Program of the Institute:

This work represents a model of yet another modality for the immunotherapy of cancer which, it is hoped, may have certain peculiar advantages. Some of these advantages are (1) it would obviate the necessity for the administration of heterologous or homologous antisera, or leukocytes. Administration of such sera or cells can sensitize to leukocyte isoantigens and may result in toxicity or secondary syndromes. The implications of these studies for the immunotherapy of human cancer are as follows. Since heterologous RNA has been shown to be effective and non-toxic, animals other than human beings could be immunized with tumor tissue and immune RNA extracted from the lymphoid tissues. Alternatively, antigen processing cells such as peritoneal macrophages or lymph node cells from such animals could be exposed, in vitro, to human tumor tissue in tissue culture, and immune RNA could then be extracted. Such RNA might be

administered to patients by direct injection by mixing the RNA with dextran sulfate. Dextran sulfate is available for clinical use in humans in clinical grades of purity. An alternative method will be to collect immunocompetent cells such as peripheral blood lymphocytes from patients by methods such as leukophoresis on the NCI cell separator. These cells could then be incubated with the "immune" RNA and then returned to the patient from which they were obtained only a short time previously. Such a model for immunotherapy would offer several theoretical advantages. First, since antigen processing is bypassed, some anergic patients might be benefited if the anergy were due to defects in antigen processing and recognition, and not due to a lack of immunocompetence of effector cells, i.e., lymphocytes. Second, this sort of immunotherapy would obviate the necessity of administering autologous or homologous tumor cells to cancer patients; (2) no adjuvant is required; (3) this model does not require the administration to patients of homologous or heterologous immunocompetent cells. Such cells contain leukocyte antigens to which the patient can be sensitized. In addition, a danger of graft versus host reaction exists. Moreover, immune elimination of such passively transferred immunocompetent cells might make prolonged immunotherapy ineffective. In our model, immune RNA would be injected directly or incubated with the patients' own autologous lymphocytes. The medicolegal problem of the source of donor leukocytes for immunotherapy is eliminated since leukocytes would not have to be obtained from other cancer patients or normal volunteers and no cross-immunization with tumor tissue would be required of other cancer patients or normal volunteers.

Proposed Course:

Efforts are now underway to isolate and characterize the active RNA in these preparations. There is evidence in the literature to suggest that such preparations may contain immunoreactive RNA-antigen complexes. Methods are being set up to attempt to isolate these RNA-antigen complexes by sedimenting our RNA preparations by ultracentrifugation in gradients of cesium sulfate. Isopycnic banding of "immune" RNA preparations in these gradients may band a unique species of RNA, possibly bound to tumor antigens. Such RNA-antigen complexes have been termed "super antigens" and are vastly more immunogenic than simple antigen alone. Complexes of such RNA with tumor antigens might behave as "super tumor antigens" and their identification, isolation, and characterization will be attempted. Further work will be directed towards investigating the direct injection of "immune" RNA mixed with dextran sulfates of various molecular weights. Dosage schedules must be worked out, the optimum molecular weight of dextran sulfate must be found. The time relationships of injection of the "immune" RNA with respect to the time of tumor cell challenge must be studied and the specificity of this reaction must be investigated in detail. In this regard, it is important to point out that since nucleic acids are, at best, very weakly antigenic, one would not anticipate that administration of "immune" RNA preparations would result in sensitization or in immune elimination of the RNA. Better methods must be found for production of "immune" RNA in larger amounts. The production of immune RNA, in vitro, by the exposure of antigen processing cells to tumor cells, in vitro, will be studied. Better methods must be found for measuring the serologic response of animals treated

with "immune" RNA and methods must be developed for quantitating the immune cytolysis produced by RNA-incubated spleen cells. A modification of the immune cytolysis method of McKhann is at present being modified for use in quantitating immune cytolysis of spleen cells incubated with immune RNA. This method uses target cells radiolabeled with tritiated thymidine. An attempt will also be made to measure the mediation of immune cytolysis of human tumor cells, in vitro, by human lymphocytes pre-incubated with "immune" RNA prepared from guinea pigs immunized to human tumors. Experiments are also planned to treat established, progressively growing tumors in rodents with immune RNA mixed with dextran sulfate and with RNA-incubated spleen cells in an attempt to cause slowing of growth or regression of such established tumors.

Objective and Approach of the National Cancer Program:

Objective 4: To prevent tumor establishment from cells already capable of forming cancers, e.g., transformed cells, cells constituting pre-cancerous tissues, and cells from primary tumors that lodge elsewhere in the body in a metastatic state, either active or dormant.

Approach 4: Interfere with the process of tumor initiation following cell transformation, including interference with development of stromal and blood vessel elements and other host responses.

Publications:

Pilch, Y.H., Ramming, K.P., and Deckers, P.J.: Transfer of tumor immunity with RNA. Israel J. Med. Sciences 7: 246-258, 1971; and In Weiss, D.W. (Ed): Immunological Parameters of Host-Tumor Relationships, Academic Press, New York, in press.

Ramming, K.P., and Pilch, Y.H.: Transfer of tumor-specific immunity with RNA: Inhibition of growth of murine tumor isografts. J. Nat. Cancer Inst. 46: 735-750-, 1971.

Deckers, P.J., Ramming, K.P., and Pilch, Y.H.: Facilitation of tumor growth by syngeneic normal spleen cells mixed with tumor cells in vitro. Cancer 27: 897-901, 1971.

Pilch, Y.H., Ramming, K.P., and Deckers, P.J.: Transfer of immunity to transplantation and tumor specific antigens by RNA from lymphoid organs of xenogeneic, allogeneic, and syngeneic animals. Trans. Proc. 3: 566-569, 1971.

Deckers, P.J., and Pilch, Y.H.: RNA-mediated transfer of tumor immunity - A new model for the immunotherapy of cancer. Cancer 28: 1219-1228, 1971.

Deckers, P.J., and Pilch, Y.H.: Transfer of immunity to tumor isografts by the systemic administration of xenogeneic "immune" RNA. Nature New Biology

231: 181-183, 1971.

IN PRESS

Deckers, P.J., and Pilch, Y.H.: Mediation of immunity to tumor-specific transplantation antigens by RNA: Inhibition of isograft growth in rats. Cancer Res.

Deckers, P.J., Edgerton, B.W., Thomas, B.S., and Pilch, Y.H.: Tumor immunity in inbred mice with progressively growing tumors - A study of concomitant immunity. Current Topics in Surgical Research.

Deckers, P.J., Edgerton, B.W., Thomas, B.S., and Pilch, Y.H.: The adoptive transfer of concomitant immunity to murine tumor isografts with spleen cells from tumor-bearing animals. Cancer Res.

Serial No. NCI-3818
1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Malignancy Associated Cellular and Serum Abnormalities

Previous Serial Number: Same

Principal Investigator: Paul B. Chretien, M.D., William F. Sample, M.D., and William J. Catalona, M.D.

Other Investigators: Bruce F. Cullen, M.D.

Cooperating Units: Anesthesiology Department, Clinical Center, NIH

Man Years:

Total:	4
Professional:	2
Other:	2

Project Description:

Part I. In Vitro Lymphocyte Reactivity and Prognosis in Cancer Patients

Part II. Correlation of Tumor Histologic Type and Clinical Stage with Cellular and Serum Abnormalities in Cancer Patients

Part III. Quantitative DNCB Reactivity in a Normal Population

Part IV. Quantitiative Dinitrochlorobenzene (DNCB) Sensitivity in Cancer Patients

Part V. Comparison of In Vivo and In Vitro Assays of Immunologic Reactivity in Cancer Patients

Part VI. Serum Ribonuclease Levels in Cancer Patients

Part VII. Peripheral Blood Macrophage Activity in Cancer Patients

Project Description:

Part I. In Vitro Lymphocyte Reactivity and Prognosis in Cancer Patients

Objectives:

To determine the relationship of in vitro lymphocyte reactivity to phytohemagglutinin in patients with non-lymphoid malignancies and prognosis after definitive surgical treatment.

Methods Employed:

The in vitro lymphocyte reactivity was determined in 94 patients who had a variety of non-lymphoid malignancies. In each, the tumor was considered clinically operable and, after determination of the lymphocyte reactivity, definitive surgical removal was attempted. The lymphocytes of 120 volunteers in good health and in the same age range as the cancer patients provided control data for the scoring of the lymphocyte reactivity of the cancer patient. Individuals with a history of malignancy, acute or chronic illness or who were taking any medication were excluded. In vitro lymphocyte reactivity was determined in autologous serum. Individual leukocyte cultures consisted of $2x10^6$ mononuclear cells in a 4 ml mixture of 80% RPMI 1640 and 20% autologous serum. Phytohemagglutinin P (PHA) was added at five different concentrations ranging from 3.1 to 50 ug of PHA/protein per ml of cultures. Triplicate cultures with each dose of PHA and controls without PHA were prepared. After 54 hours incubation, attritiated thymidine was added and at 72 hours, the cultures were terminated. The nuclear protein was precipitated with trichloroacetic acid and the precipitated process for scintillation counting. The radioactivity of each culture was determined in a Model 303 Packard Tri-Carb scintillation counter. The results were corrected for quench and expressed in disentigration per minute (DPM).

Major Findings:

To derive an age-correlated range of normal lymphocyte reactivity, the 120 control subjects were divided into five groups of approximately equal age intervals and the mean reactivity ± 1 standard deviation (SD) determined for each group. The lymphocyte reactivities of the control population have a normal distribution, i.e., 84 patients (70%) had lymphocyte reactivity within ± 1 SD of the means and 18 patients (15%) each had reactivity greater than and less than 1SD of the mean. The highest lymphocyte reactivity occurred in the youngest individual studied. From a peak in the second decade, reactivity declined over the next four decades, then was relatively constant through the last decade studied.

Three years after completion of the determination of lymphocyte reactivity of the cancer patient, the medical records of the patients were reviewed.

Follow-up was complete on all patients. The patients were classified either as: (1) clinically cured of tumor at the time of the review (34 patients); (2) as inoperable (29 patients); or (3) as having recurrent tumor - either at the site of operation or in the form of distant metastases - after tumor resection that was considered complete (31 patients). The lymphocyte reactivity of each patient was arbitrarily defined in relation to the reactivity of the control population. The reactivity within ± 1 SD of the mean age-correlated control reactivity was classified as normal; reactivity greater than 1SD of the mean control reactivity was classified as high and reactivity less than 1SD of the mean control reactivity was classified as low. The distribution of the lymphocyte reactivity of the cancer patients were significantly lower than that of the controls. The difference is due primarily to the percent of cancer patients with low lymphocyte reactivity (45% compared with 15% of controls). Significantly fewer cured cancer patients, however, had low lymphocyte reactivity than did inoperable patients or patients with recurrent tumor. There was a significantly greater percent of cured patients with inoperable or recurrent tumors (7%).

Significance to Biomedical Research and Program of the Institute:

The relationship shown in this study of *in vitro* lymphocyte reactivity to extent of tumor growth and prognosis after surgical treatment shows that this test assays a clinically significant aspect of the host-tumor relationship. The studies which demonstrate a correlation between the delayed hypersensitivity response to dinitrochlorobenzene and prognosis in cancer patients and those which show that the DH response to DNCB parallels *in vitro* lymphocyte reactivity when considered together, suggest that *in vitro* lymphocyte reactivity provides similar incite into the prognosis of cancer patients as DH to DNCB. These two tests combined, therefore, may have increased value as the means of predicting the course of cancer patients who receive definitive treatments of their malignancies.

Proposed Course:

Continued improvement in this assay system to provide a more quantitation of *in vitro* lymphocyte reactivity and extension of the assay to include patients clinically cured of tumor and continued assay of patients studied preoperatively will further determine the relationship of *in vitro* reactivity to susceptibility to cancer, effect of cancer treatment, and patient feasibility for adjunctive therapy which operates through alteration of immunologic responsiveness.

Project Description:

Part II. Correlation of Tumor Histologic Type and Clinical Stage with Cellular and Serum Abnormalities in Cancer Patients

Objectives:

To quantitate abnormalities of *in vitro* lymphocyte reactivity and serum effects on lymphocyte reactivity in cancer patients and determine the incidences of these abnormalities in patients with varied histologic types of tumors and the relationship of tumor stage.

Methods Employed:

Preoperative patients with non-lymphoid tumors of a variety of histologic types were studied. Controlled data was obtained from healthy volunteers in the same age as the cancer patients. Volunteers with a history of chronic disease, medication ingestion, recent illness or history of malignant tumor were excluded. An AB serum pool, prepared from healthy volunteers, was used as the constant reference source for both cellular and serum reactivity. Purified specimens of mononuclear cells were obtained from the cancer patients and the controls by the exposure of whole blood to carbonyl iron and separation of the cells that phagocytize the iron particles. Cultures containing $5x10^5$ mononuclear cells/ml of 90% RPMI 1640 and 10% AB pooled serum were prepared. Phytohemagglutinin-P (PHA) was used in five concentrations to determine the peak response to PHA. Cultures with each dose of PHA and controls without PHA were prepared in triplicate. After a culture interval of 24 hours, tritiated thymidine was added for a three hour labelling period, after which the cultures were terminated. The cultures were processed for scintillation counting and the radioactivity expressed in disintegration per minute (DPM).

Major Findings:

The cellular reactivity of the cancer patients was arbitrarily defined in terms of the response of normal volunteers: It was normal if it was within 1SD of the mean for the response of volunteers of the same age group; high if it exceeded the mean by more than 1SD; and low if it was less than 1SD of the mean. As a group, significantly more patients with cancer had low reactivity than controls (30% versus 16%). When lymphocyte reactivity was correlated with histology, the highest incidence of low reactivities was seen in patients with squamous carcinoma (45%) and sarcoma (41%). Patients with melanomas and adenocarcinomas as a group had the same lymphocyte reactivity as the control patients. When the patients were divided into three groups according to the stage of tumor growth, there was no correlation between tumor stage and lymphocyte reactivity. Using the AB serum pool as a constant reference, defect of sera from cancer patients on the reactivity of normal lymphocytes showed that serum from patients with squamous and sarcoma significantly inhibited the reactivity of normal lymphocytes. Serum from patients with melanomas and adenocarcinomas did not display this abnormality. Serum and cellular abnormalities coexisted in patients with sarcoma but occurred independently with patients with squamous carcinoma.

Significance to Biomedical Research and Program of the Institute:

A major finding in this study is the association of a greater incidence of low lymphocyte reactivity in patients with tumors of certain histologic types. This information is invaluable in planning immunotherapy regimens that attempt to correct immunological defects. Second, the relationship of low lymphocyte reactivity to development of tumor may be pertinent to the epidemiology and control of cancer. If the low lymphocyte reactivity preceded the tumor in these patients, assays of this type may be useful as a means of screening for cancer-prone individuals and detection carcinogenic environmental conditions. If low lymphocyte reactivity is the consequence of cancer, whether reversion to higher reactivity occurs with clinical cure should be determined. If it does, assay of lymphocyte reactivity may be a sensitized means of monitoring effects of tumor treatment. The results of this study indicate that only patients with certain histologic types of tumors can be monitored in this manner. The demonstrated relationship of serum suppression factors to tumor histologic type also provide a new, rational basis for the design of immunotherapy treatment protocols based on tumor histology. Failure to demonstrate serum suppressants of lymphocyte reactivity in patients with melanomas and adenocarcinomas may indicate that tumor vaccine treatment for these patients are less likely to have the undesirable affect of suppressing immunity by invoking the production of these serum factors than in patients with epidermoid carcinomas and sarcomas.

Proposed Course:

Studies in progress will determine the nature of the serum inhibitory factor demonstrated and those factors which elicits its production. Control of the action of this factor is of obvious importance in immunologic approach to irradication of malignancy. The effect of immunological adjuvants on the alteration of the suppressed lymphocyte response of this is also being studied.

Project Description:

Part III. Quantitative DNCB Reactivity in a Normal Population

Objectives:

To derive age-correlated data concerning normal DNCB reactivity determined with a method that quantitates allergy to DNCB.

Methods Employed:

Because of the limitations of previous methods of determing delayed hypersensitivity, which score patients as either anergic or allergic, a scheme for quantitating allergic response to dinitrochlorobenzene (DNCB) was devised. A 2000 µg dose of DNCB was applied to the upper arm simultaneously with the application of 50 µg to the volar forearm of normal individuals.

The subjects were examined daily for a minimum of 21 days. A spontaneous flare reaction at both the 2000 µg and 50 µg sites by the 14th day is graded 4+. A spontaneous flare at the 2000 µg site alone is graded 3+. In the absence of a spontaneous flare at the 2000 µg site, a challenge dose of 50 µg is applied on the 14th day. An unequivocal DCH response after 48 hours is graded 2+. Unequivocal reactions are biopsied. If these show a mononuclear cell infiltrate, the reaction is graded 1+. Two months after completion of the above studies, additional subjects were selected for the study of the threshold of DNCB necessary to elicit an anamnestic response. Correlations were made between the reactivity observed with the primary response and the dose of DNCB required to elicit an anamnestic response.

Major Findings:

Of the 143 subjects tested, 139 (97%) were sensitized to DNCB by the criteria used. All but one of the sensitized subjects exhibited either a 3+ or 4+ reaction. The mean interval between sensitization and a spontaneous flare reaction was 9-10 days for the 2000 µg and 11-12 days for the 50 µg dose. There was no correlation between age and the positive response to DNCB. Four subjects were anergic and although they were all over 40 years of age, the ages were equally distributed through the last four age decades studied. Subjects who had primary 4+ reactions developed an anamnestic response to DNCB challenge with a mean dose of 5 µg of DNCB. Subjects who had 3+ reactions responded to a mean dose of 25 µg of DNCB. Although a trend toward higher reactivity was observed in patients with a history of allergy, no statistically significant difference could be established. Similarly, no correlation could be demonstrated between reactivity to DNCB and race, family history of cancer, the use of alcohol or the use of tobacco.

Significance to Biomedical Research and Program of the Institute:

While sensitive techniques have been developed for the quantitation of humoral antibody responses, methods of measuring cell-mediated immunity are crude by comparison. The elicitation of delayed cutaneous hypersensitivity reactions to intradermally injected antigens has often been implored widely to measure cell-mediated immunity in vivo. However, these antigens yield limited information concerning cellular immune responsiveness since most are complex biologic extra-ts which incite a combination of both humoral and cellular immune responses. Furthermore, they are widely employed to elicit anamnestic responses which rely upon previous exposure to respond to a newly-encountered antigen. The limitations of intradermal DCH tests are obviated by the use of dinitrochlorobenzene. The agent is an allergen of definite known constitution, therefore, sensitization and challenge can be accurately controlled in terms of time and dose and experimental conditions are exactly reproducible. Humoral antibodies are not involved in DCH reactions to DNCB, thus, it is a specific test for cell-mediated immunity that assesses the current ability of a subject to manifest DCH to newly encountered antigen. This method of quantitating DCH to DNCB, therefore, provides the first method of quantitating in vivo immunologic responsiveness

and will be invaluable in assessing the immunologic status of cancer patients before and after treatment and in quantitating the effects of therapy regimens on immunologic responsiveness.

Proposed Course:

This method of quantitating DCH to DNCB was used to measure the immunologically responsiveness of all patients admitted to the Surgery Branch for definitive cancer therapy. Studies of patients cured of cancer are also in progress to determine the relationship of the abnormalities demonstrated preoperatively to prognosis.

Project Description:

Part IV. Quantitative Dinitrochlorobenzene (DNCB) Sensitivity in Cancer Patients

Objectives:

To quantitate in vivo immunologic responsiveness in patients with non-lymphoid tumors using an assay method that quantitates delayed hypersensitivity (DH) to DNCB.

Methods Employed:

All patients with non-lymphoid tumors admitted to the Surgery Branch for definitive treatment of their malignancies undergo preoperative assay of DH to DNCB. The method employed incorporates the spontaneous flare reaction which occurs to the residual DNCB remaining in the skin after sensitization. A spontaneous flare at the 2000 µg site and the 50 µg sites by 14 days after sensitization is graded 4+. A spontaneous flare at the 2000 µg site only is considered 3+, and a response elicited only after challenge with 50 µg is considered 2+. Reactions that require biopsy to demonstrate are graded 1+. The reactivities of the cancer patients were correlated with tumor histologic type and clinical stage.

Major Findings:

Only 41% of cancer patients developed spontaneous flare reactions to DNCB, an incidence that is significantly less than in controls (96.5%). An additional 29% required challenge to elicit DCH to DNCB and 30% were anergic. When the patients were grouped according to histologic type of tumor present, all groups had significant abnormal reactivity. The incidence of anergy was highest in patients with squamous carcinoma (43%) less in patients with melanomas (25%) and adenocarcinomas (26%) and not significantly different from controls in patients with sarcomas (12%). Of patients with squamous carcinoma, 28% had impaired positive reactivity which when combined with the incidence of anergy made them the most abnormal group. The impaired positive reactivity in patients with melanoma (44%) and adenocarcinomas (26%) further magnify the

abnormality of their expected reactivity. In patients with sarcomas, among whom the proportion of anergy was not significantly different from controls, the 25% incidence of impaired positive reactivity rendered the distribution of their reactivities significantly abnormal when compared with controls. When the patients were divided into two groups according to the extent of tumor determined preoperatively and at operation, and those with localized tumors and those with metastatic tumors, there was no statistically significant differences in the distribution of reactivities in the two groups. When considered by tumor histologic type, patients with sarcoma showed a relationship between impaired reactivity to DNCB and extent of tumor present.

Significance to Biomedical Research and Program of the Institute:

The most important contribution of this assay method is the impaired positive reactivity (2+ and 1+) revealed in allergic cancer patients. These levels of impaired reactivity occurred in a high incidence among patients with tumors of all histologic types studied, thus significantly increasing the proportion in each group with abnormal reactivity. That the incidence of abnormal reactivities was highest in patients with squamous cancer, significantly less in patients with melanoma and adenocarcinoma and least in those with sarcoma, yet with exception of sarcomas, was not related to clinical extent of tumor, incidates differing host-tumor relationship exists which are determined by the histologic type of tumor present. Therefore, in investigations of cellular immune responses of cancer patients, it is essential to consider tumor histology. This study of cancer patients and the previous study of normal controls demonstrates the method of quantitating DH to DNCB distinguishes at least three levels of reactivity (4+ and 3+, 2+ and 1+, and anergy) which appear to be biologically meaningful.

Proposed Course:

The subsequent clinical courses of the patients entering this study will eventually show the relationship of the levels of DH to DNCB demonstrated and prognosis of cancer patients who receive definitive treatment of their tumors. The assay method is also incorporated into an immunotherapy protocol which selects patients on the basis of immunocompetence. The assay will also be used to monitor the effects of the treatment on immune responsiveness.

Project Description:

Part V. Comparison of In Vivo and In Vitro Assays of Immunologic Reactivity in Cancer Patients

Objectives:

To compare in vitro cellular immunity as assayed by lymphocyte response to phytohemagglutinin (PHA) and in vivo immunologic responsiveness assayed by quantitated contact sensitivity to dinitrochlorobenzene (DNCB).

Methods Employed:

Patients admitted to the Surgery Branch who had clinically operable malignancies were selected for study. All patients were in good general health and had no chronic or acute disease, and were taking no medications. No patient had undergone general anesthesia, radiotherapy, chemotherapy or steriod therapy for at least three months prior to testing. DNCB contact sensitization was determined using the quantitative scheme developed by this unit. This scheme measures four distinct levels of positive responsiveness as outlined elsewhere in this section. In vitro lymphocyte responsiveness to phytohemagglutinin in short-term tissue culture was determined by quantitation of tritiated thymidine incorporation. The cells were cultured in homologous AB serum to obviate the effect of serum inhibitors of lymphocyte reactivity.

Major Findings:

The lymphocyte reactivity index was determined by dividing the lymphocyte reactivity in disintegrations per minute (DPM) for the patient by the mean reactivity (DPM) for normal individuals of the same age groups. Lymphocyte reactivity was then expressed as a function of in vivo DH to DNCB. Analysis of this data yielded a correlation coefficient of .72 which is significant at the 95% level of confidence. Both anergic and maximally responsiveness as assessed by DNCB reactivity displayed a range of lymphocyte responsiveness, however, anergic patients as a group have the lowest lymphocyte reactivity and maximally sensitive patients had the highest lymphocyte reactivity.

Significance to Biomedical Research and Program of the Institute:

This study demonstrates that lymphocyte in vitro reactivity to PHA parallels DH to DNCB, but each assay contributes unique data. Lymphocyte reactivity to PHA further quantitates cellular reactivity at the extreme of DH to DNCB and also reveals serum inhibitors of cellular reactivity as described elsewhere in this section. DH to DNCB tests in vivo cellular mechanisms only but does so in the presence of serum inhibitors. Therefore, the utilization of both assays provides complimentary data concerning the immunologic reactivity of cancer patients. Because of the mounting evidence that the immunologic reactivity of cancer patients determines the results of treatment and that the alterations in immunologic responsiveness induced by chemotherapy and immunotherapy correlate with the effects of such therapy, it is increasingly important that the immunologic reactivity of cancer patients be accurately determined. This study demonstrates that these two assays are effective in this goal.

Proposed Course:

With the validity of both of these assays established as methods that quantitate immunologic reactivity, both will be used to quantitate reactivity prior to the institution of definitive therapy and to monitor the effects of therapies, especially chemotherapy and immunotherapy.

Project Description:

Part VI. Serum Ribonuclease Levels in Cancer Patients

Objectives:

To determine the significance of elevated serum ribonuclease levels in cancer patients. To relate this abnormality with tumor histologic type and stage.

Methods Employed:

Serum alkaline ribonuclease levels were measured by spectrophotometric quantitation of soluable products of RNA digestion by serum ribonucleases. Optimum activity occurred at Ph7.8. AB pooled serum specimens from a single source was used as a constant reference point. Age-correlated criteria of normal ribonuclease levels were determined through a study of serum from over 200 normal subjects. The sera of over 90 cancer patients with a variety of histologic types and stages of malignancies were similarly assayed. All cancer patients were in otherwise good health. Patients with a history of chronic disease or who were taking medications were excluded.

Major Findings:

A significant correlation between serum ribonuclease levels and tumor histologic type was found. Patients with melanomas and sarcomas had ribonuclease levels which as a group did not differ from that of normal controls. Patients with squamous carcinomas and adenocarcinomas, however, had significantly elevated ribonuclease levels. There was no correlation between tumor stage and ribonuclease levels in patients with these latter two types of tumors.

Significance to Biomedical Research and Program of the Institute:

This study demonstrates that patients with squamous carcinomas and adenocarcinomas have significantly elevated alkaline ribonuclease levels. Further study of this phenomenon through assay of the same patients after definitive treatment of their tumors and assay of patients clinically cured of cancer will determine the significance of these findings. If these studies show a return to normal levels after tumor removal of the ribonuclease measured, the use for this assay as a tumor "marker" would appear feasible. Patients with elevated ribonucleases that persist after tumor removal would therefore suggest continued presence of tumor. In such patients, this assay would allow the earlier institution of adjuvant therapy for subclinical residual disease.

Proposed Course:

Continued study of the serum of these patients after definitive tumor treatments and assay of patients clinically cured of tumor are in progress to determine the usefulness of this assay as an indicator of clinically occult tumor.

Project Description:

Part VII. Peripheral Blood Macrophage Activity in Cancer Patients

Objectives:

To quantitate peripheral blood macrophage phagocytosis and enzymatic processes in cancer patients.

Methods Employed:

A technique for isolation of blood phagocytes (neutrophils and monocytes) was developed. Phagocytic activity was quantitated by the determination of latex polystyrene particles phagocytosis. Enzymatic digestive mechanisms were quantitated by reduction of p-nitro tetrazolium blue (NBT) in response to latex particle ingestion. Cancer patients who were in otherwise good general health were studied. None had other chronic diseases and all were afebrile and had normal peripheral blood cell counts. Normal controls in the same age group were studied to establish normal control values for these assays.

Major Findings:

All cancer patients studied demonstrated abnormalities of phagocytosis or intracellular enzymatic reduction of NBT. Patients with squamous carcinoma were the most abnormal group with severely suppressed phagocytosis at all doses of latex used. NBT reduction was similarly suppressed to extremely low levels. Patients with sarcoma displayed the same abnormalities but to a lesser degree. Patients with melanomas had normal latex phagocytosis but significantly suppressed reduction of NBT. There was no correlation between tumor stage and the abnormalities demonstrated.

Significance to Biomedical Research and Program of the Institute:

This investigation reveals hitherto to unappreciated abnormalities in cancer patients. The mounting evidence that macrophage processing of tumor antigen is a necessary prerequisite for immunologic response indicates that defects in phagocytosis may account for impaired host resistance to cancer. The studies of this aspect of immune mechanism should, therefore, provide significant incite into the host-tumor relationship. In addition, therapy regimens should be devised that correct this abnormality.

Proposed Course:

Continued study of all patients prior to treatment of their malignancy by this assay method will be done. In addition, these patients will be studied at intervals postoperatively to determine whether these defects preceded the development of cancer and whether a persistent abnormality of macrophage activity is indicative of persistent tumor. The assay is also being incorporated into an immunotherapy regimen. Preliminary observations indicate that BCG stimulates macrophage activity. The response of macrophages to BCG may therefore select those patients likely to benefit from this form of immunotherapy.

Objective and Approach of the National Cancer Program:

Objective 5: To achieve an accurate assessment of the presence extent and probable course of cancer risks in population groups (including attention to precancerous lesions) and of cancers in individuals alone (diagnosis) and in groups (detection) as an aid to prevention cure, or prognosis.

Approach 4: Improve diagnostic procedures for cancer and high and low risks. For localization of lesions.

Publications:

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In Press:

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3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Study of Cancer Immunology and Immunotherapy in Laboratory Animals

Previous Serial Number: Same

Principal Investigator: George H. Myers, Jr., M.D.

Other Investigators: D.L. Morton, M.D., E.C. Holmes, M.D., F.R. Eilber, M.D., P.J. Deckers, M.D., B.W. Edgerton, M.D., Y.H. Pilch, M.D., E.E. Fraley, M.D., S. Ecker, M.D., J.B. DeKernion, M.D., W.J. Pendergrast, Jr., M.D., and M.S. Soloway, M.D.

Cooperating Unit: Laboratory of Pathology, NCI

Man Years:

Total:	4-1/2
Professional:	2
Other:	2-1/2

Project Description:

Part I. Intradermal Immunization Against Transplantable Tumors in Inbred Mice

Part II. The Effect of Neuraminidase on the Immunogenicity of Viral and Chemically Induced Tumors in Mice and Guinea Pigs

Part III. The Effect of Immunosuppression and the Mammary Tumor Virus on the Growth of Transplanted Mouse Mammary Tumors

Part IV. The Effect of Homologous Mammary Tumor Immunizations on Spontaneous and Transplantable Mammary Tumors

Part V. Search for a Humoral Antitumor Antibody in the Sera of Guinea Pigs Undergoing Immunotherapy

Part VI. The Influence of Regional Lymphatics on Tumor Immunity

Part VII. The Effect of Regional Lymphadenectomy on Concomitant Tumor Immunity

Part VIII. The Immunogenicity of a Murine Renal Adenocarcinoma

Part IX. Hormonal and Chemotherapy for Renal Cell Carcinoma

Part X. The Production of Bladder Tumors in Inbred Mice and the Study of their Immunologic Properties

Part XI. Tissue Culture Studies of Human Prostatic Tissues

Project Description:

Part I. Intradermal Immunization Against Transplantable Tumors in Inbred Mice

Objectives:

This study was designed to determine whether or not it is possible to significantly inhibit the growth of mouse tumors which have been inoculated intramuscularly into isologous hosts by the concomitant intradermal immunization with syngeneic tumor. This study was stimulated by the findings in guinea pigs, using a chemical induced sarcoma and a hepatoma, that repeated intradermal immunizations with viable tumor cells was effective in preventing the growth of intramuscular tumor challenge. It was the purpose of this study to see if mice undergoing similar immunization also could be protected against tumor growth.

Methods Employed:

Using an inbred mouse system, an attempt has been made to evaluate the effects of immunotherapy with two different tumors. A methylcholanthrene induced sarcoma in C57Bl/6N mice was chosen as an example of a carcinogen induced tumor, and a spontaneous mammary tumor from a C3H/HeN mouse was chosen as an example of a viral induced tumor. The intradermal route was chosen for immunotherapy since it is thought that the skin confers a greater immuno-reactive capability than subcutaneous and intramuscular sites. The minimal cell dose which would produce tumor in 80-100% of animals was determined for each tumor prior to initiating this study. After a known tumor producing cell inoculum was injected intramuscularly into the hind limb of isologous host, a non-tumor producing inocula of viable tumor cells was injected intradermally into several sites of the same animal. A corresponding group of animals also received intradermal injections of irradiated tumor cells.

Major Findings:

Intradermal immunization with irradiated tumor cells has not been effective in preventing the growth of intramuscular tumor challenge unless the challenge was extremely small. However, immunotherapy against tumor cells injected intramuscularly has been accomplished by the concomitant intradermal immunization with viable tumor cells. This success has only been found in the case of the most antigenic methylcholanthrene induced sarcoma. So far, no inhibition of the viral induced mammary carcinoma has occurred using such an immunotherapy model. The effects of the intradermal immunizations in the mice, using the methylcholanthrene induced sarcoma, is less pronounced than in the guinea pig. This was originally thought to be due to the guinea pig's superior capability in developing a cellular immune response. However, further studies in our laboratory have detected an antibody in the serum of guinea pigs undergoing repeated intradermal immunizations against a methylcholanthrene induced sarcoma.

Significance to Biomedical Research and Program of the Institute:

Further studies in experimental animals are necessary before immunotherapy can be applied to human cancers. Preliminary studies suggest that small numbers of tumor cells can be prevented from growing by the intradermal immunization with viable tumor cells. Although immunotherapy of cancer may not be beneficial as a primary form of treatment, it may become useful as a supplement to the more conventional forms of treatment, such as surgery and chemotherapy.

Proposed Course:

These studies have been continued using other methylcholanthrene-induced tumors to further evaluate the effectiveness of intradermal immunotherapy and/or immunoprophylaxis both as primary treatment as an adjunct to surgery and/or chemotherapy.

Project Description:

Part II. The Effect of Neuraminidase on the Immunogenicity of Viral and Chemically Induced Tumors in Mice and Guinea Pigs

Objectives:

It has been suggested by other authors that the effective antigenic expression by some types of tumor cells may be inhibited by the presence of sialic acid on the cell periphery. The treatment of several non-specific transplantable mouse tumors with neuraminidase has resulted in an apparent increase in their immunogenicity. Little of the information so far obtained has been related to the growth and development of autologous tumors and their antigenicity in isologous hosts. This project was undertaken to study the effects of neuraminidase on the immunogenicity of both a chemically and a viral induced tumor in isologous hosts.

Methods Employed:

A methylcholanthrene-induced sarcoma in C57Bl/6N mice is being used as an example of a carcinogen induced tumor, and a spontaneous mammary tumor from a C3H mouse as a viral induced tumor. Tumor cells are incubated with the enzyme neuraminidase which has been obtained from cultures of Vibrio cholerae. Following incubation the tumor cells are resuspended in media and injected in various concentrations into isologous animals. When tumors fail to grow, the animals are rechallenged intramuscularly with a known tumor producing inocula and their immunity measured by low incidence of tumor development, a delay in tumor appearance, slower growth rate, and prolongation of survival time as compared to control animals. In addition, immunotherapy studies are being performed using neuraminidase treated cells injected intradermally in order to protect against an intramuscular tumor challenge.

Major Findings:

When tumor cells are incubated with neuraminidase at concentration of 500 units per ml in 0.05 molar sodium acetate acitic acid buffered saline at pH 5.5 and containing calcium ions, there is a loss of viability (determined by dye exclusion studies) in the magnitude of 30-50%. The acetate buffer alone has been found to cause the same loss of viability of tumor cells. A methylcholanthrene induced sarcoma which usually grew at $1x10^3$ cells failed to grow at $2x10^6$ after the cells were treated with neuraminidase. However, when these animals were challenged, they failed to demonstrate immunity. When tumor cells were suspended in a buffer of pH 7.0 and incubated with neuraminidase there was no alteration of tumor growth even at the low cell dose as compared to untreated cells. A similar experience has been obtained with a viral induced mammary tumor. So far, we have not been able to show an increase in immunogenicity with either tumor after treatment with neuraminidase. However, using neuraminidase treated cells for intradermal immunotherapy, it has been possible to significantly prevent the growth of an intramuscular tumor challenge. This protection is no greater than that seen using viable cells but seems to be greater than that seen using irradiated cells. When the guinea pig was used for intradermal immunizations using neuraminidase treated cells, there was less protection when the cells were so treated than when viable tumor cells were used as the immunogen.

Significance to Biomedical Research and Program of the Institute:

If it is true that some tumor cells have the masking of the antigenic expression by sialic acid and that neuraminidase can increase their antigenicity, then it is conceivable that the treated cells can be used for immunotherapy of cancer. Similarly, it could be that such a sialomucin coating of the cell surface prohibits the host animal from mounting an effective immune response to cancer.

Proposed Course:

Despite the fact that our findings are not as encouraging as reported by others using other tumor systems, we are going to continue to study the effects of neuraminidase as a possible adjunct to immunotherapy with particular attention to routes of immunization and comparison of other forms of immunization.

Project Description:

Part III. The Effect of Immunosuppression and the Mammary Tumor Virus on the Growth of Transplanted Mouse Mammary Tumors

Objectives:

During the past several years a number of reports have supported the hypothesis that some and perhaps all mouse mammary carcinomas induced by the mammary tumor virus are antigenic in syngeneic mice or even in the autochthonous host. However, the presence of the mammary tumor virus markedly alters the results of

immunization studies. Sublethal irradiation of recipient mice has been shown to increase the growth of transplanted mammary carcinomas only when the animals were free of the mammary tumor virus (MTV-free). This project is being designed to study if other methods of immunosuppression similarly alter the resistance against tumor challenge, and to what extent the presence or absence of the mammary tumor virus affects the resistance.

Methods Employed:

Spontaneous mammary tumors from C3H/HeN and Balb/c mice will be used for these experiments. Both strains of mice will contain sublines of virus-infected and virus-free mice of the same inbred strain. Methods of immunosuppression will consist of total body irradiation, cortisone and rabbit-antimouse lymphocyte serum. Each subline will be challenged with tumor following immunosuppression. It may be assumed that increased resistance against tumor challenge in mice without the mammary tumor virus is on an immunological basis if the various forms of immunosuppression reduce the resistance of the MTV-free mice against tumor challenge, but has little or no effect on those mice congenitally infected with the mammary tumor virus. If immunization actually allows the virus-infected mice to increase their resistance to tumor, then there is a suggestion that acquired immunological tolerance may be partially broken.

Major Findings:

Mice lacking the mammary tumor virus when challenged with syngeneic mammary tumor following immunosuppression had an increased incidence of tumor growth. Animals positive for the mammary tumor virus have not shown increased growth of syngeneic tumor while they are immunologically suppressed and even at times have shown a slightly slower growth rate than the controls.

Significance to Biomedical Research and Program of the Institute:

Preliminary observations with total body irradiation suggest that the acquired immunological tolerance of mice infected with the mammary tumor virus may be partially broken. If tolerance proves to be important for effective carcinogenesis by other oncogenic viruses in nature, than other means for breaking this tolerance will be needed if immunotherapy of viral-induced cancer is to be effective.

Proposed Course:

A search for means of breaking immunologic tolerance is presently underway.

Project Description:

Part IV. The Effect of Homologous Mammary Tumor Immunizations on Spontaneous and Transplantable Mammary Tumors

Objectives:

The purpose of this study was to determine if homologous mammary tumor could successfully prevent the occurrence of spontaneous mammary tumors or inhibit the transplantation of syngeneic mammary tumor cells. The purpose of using homologous rather than isologous tumor for immunization was in hope of being able to use higher concentrations of tumor cells with perhaps better immunization.

Methods Employed:

Spontaneous mammary tumors from the inbred strains of mice C3H/Hen and Balb/c were used for these experiments. Both strains of mice will contain sublines of virus infected and virus-free mice of the same strain. Following repeated subcutaneous and/or intraperitoneal injections of viable homologous tumor cells, animals will be challenged with syngeneic mammary tumor. X-breeder female mice from the inbred strain C3H/HeN will undergo repeated immunizations with either homologous mammary tumor, homologous methylcholanthrene-induced sarcoma, and normal tissues from homologous Balb/c mice.

Major Findings:

Repeated immunizations with homologous mammary tumor failed to give significant protection to transplanted syngeneic tumors. In fact, in some instances there seemed to be enhanced tumor growth. The presence or absence of the mammary tumor agent seemed to have little effect on the response to these immunizations. On the other hand, X-breeder females undergoing repeated immunizations with homologous mammary tumor tissue had a significant increase in the incidence of spontaneous mammary tumors. This was not seen when either normal tissues or another tumor was used for immunization.

Significance to Biomedical Research and Program of the Institute:

These studies suggest that the use of homologous mammary tumor is not effective as a means of immune prophylaxis against either spontaneous mammary tumors or transplantable tumors.

Proposed Course:

These studies are being repeated and should be completed soon.

Project Description:

Part V. Search for a Humoral Antitumor Antibody in the Sera of Guinea Pigs Undergoing Immunotherapy

Objectives:

It has now been shown that guinea pigs can be protected from intramuscular tumor challenge if they undergo repeated intradermal injections of viable tumor cells as well as antigens prepared from tumor cells. Although we have found similar findings in mice the effect is much less striking than in the guinea pig. The results in the guinea pig have prompted other investigators to begin immunizing humans with autologous or homologous tumor cells. Patients so immunized have been found to contain an antibody which is thought to be specific for the immunizing tumor. The success with the immunotherapy studies in the guinea pig has previously been thought to be due to a cellular or delayed type of hypersensitivity response. Until now, guinea pigs so immunized have not been shown to contain anti-tumor antibody. It was the purpose of this study to see whether or not we could define by means of the complement fixation technique an antibody in the sera of guinea pigs undergoing intradermal immunization against a methylcholanthrene-induced sarcoma.

Methods Employed:

Inbred guinea pigs of strain 2 were used for this study. Methylcholanthrene induced sarcomas designated MCA-A and MCA-25 were the tumors used. Immunotherapy consisted of multiple intradermal injection of a subtumor growth inocula following an intramuscular tumor challenge.

The animals were serially bled by the intracardiac route, their serum stored and later examined by modification of the microtiter complement fixation technique for the presence of anti-tumor antibody. Soluble tumor antigen was prepared from a tissue culture cell line of the MCA-A or MCA-25 tumor as well as from the freshly excised tumor.

Major Findings:

Intradermal immunizations, using irradiated and unirradiated tumor cells, have been shown to be effective in preventing intramuscular tumor growth in syngeneic tuinea pigs. The sera of animals so immunized have been found to contain an antibody that reacts with the MCA-A (in this case the immunizing tumor) antigen and does not react with antigens prepared from the MCA-25, normal Strain 2 guinea pig fibroblast grown in tissue culture, or normal Strain 2 liver muscle or heart tissues. Further demonstration of the specificity of the reaction was obtained by utilizing serum from animals immunized to media 1640, fetal calf serum, and Gey's solution with 0.25% trypsin. None of these sera gave a positive complement-fixation technique when reacted with the MCA-A antigen. The anti-tumor antibody found in the sera of these guinea pigs seems to be of the 19-S variety since the complement-fixation reaction is destroyed by pretreating the sera

with mercaptoethanol. Using guinea pig sera with a positive complement-fixation titer, we have been able to passively transfer the immunity to other Strain 2 guinea pigs and prevent the growth of the MCA-A tumor.

Significance to Biomedical Research and Program of the Institute:

These studies have demonstrated for the first time an antibody in guinea pigs immunized against a carcinogen induced tumor. Previous studies that have detected an antibody against a carcinogen induced tumor have been solely confined to the murine system. Our findings seem to correlate well with the findings that have been recorded from human immunizations. This may become an important model for future immunotherapy. This will allow better characterization of the tumor antigens associated with carcinogen induced tumors. These findings suggest that a humoral response is important in tumor prevention as well as a cellular immune response.

Proposed Course:

These studies will be continued using other tumors in both guinea pigs and mice. Attempts will be made to further characterize the antibody and the antigen involved.

Project Description:

Part VI. The Influence of Regional Lymphatics on Tumor Immunity

Objectives:

The regional lymphatics have been shown to play an important role in the rejection of skin allografts. This has been best shown by Barker and Billingham using alymphatic skin pedicles on the flanks of guinea pigs. Skin allografts on such pedicles were found to survive indefinitely. Animals with a pedicle graft in place will reject the other graft from the same donor in a first-set fashion. Not only is the second graft rejected, but the first graft on the pedicle is also rejected thereby establishing the presence of intact efferent arc of the immune system. We propose to use a similar model to study the effects of regional lymphatics on tumor allografts as well as tumor isografts.

Methods Employed:

Two methylcholanthrene tumors will be used in this study; one designated MCA-A, the other designated MCA-25. Both of these tumors have originated in Strain 2 guinea pigs and will serve both as allografts and isografts. Strain 2 and Strain 13 animals will be used. Alymphatic skin pedicles will be created on the flanks of these animals and the pedicles challenged intradermally with various tumor cell doses. This same model will be used to study immunotherapy and adoptive transfer of tumor immunity in guinea pigs as well as the effects of BCG on tumor growth.

Major Findings:

Tumor allografts injected into alymphatic skin pedicles have been found to grow to as large as 3 cm, but all eventually regress. The size of the tumor growth in the pedicles is considerably larger than any tumor produced from allogeneic tumor cells in the intact skin. Animals previously bearing a tumor allograft, if rechallenged, demonstrate immunity and this immunity can be transferred to other animals by the use of spleen cells. In contrast, tumor isografts in alymphatic pedicles grow to as large as 6 cm, resulting in death of the animal. This can be shown to occur with a cell dose which would never produce a progressively growing tumor in the intact skin. If a pedicle bearing a tumor isograft is excised early in the course of its growth and the animal rechallenged with syngeneic tumor he also demonstrates immunity. These findings suggest that the regional lymphatics are important for the early initiation of tumor growth but that once this tumor obtains a certain size it no longer is under the influences of the local immune system. In spite of systemic sensitization, the animal is unable to reject a tumor when only weak tumor specific antigens are present.

Significance to Biomedical Research and Program of the Institute:

Recently, a few clinicians have been advocating leaving the local lymph nodes in place during cancer surgery in order to preserve the patient's immune defenses. Our findings would suggest that once a tumor is large enough to be clinically detected, the patient has already been sensitized systemically and would not benefit by leaving the local lymph nodes intact, since many of these may contain metastatic tumor.

Proposed Course:

Further investigation will be carried out using this animal model system in hopes that a better understanding of the regional lymphatics can be obtained.

Project Description:

Part VII. The Effect of Regional Lymphadenectomy on Concomitant Tumor Immunity

Objectives:

With the increasing evidence of immunologic factors associated with the initiation and progression of both human and animal tumors, much recent attention has been focused on the role of the regional lymph nodes in the expression of host ability to resist tumor growth. Drs. Crile and Fisher have both suggested that regional lymphadenectomies significantly alter the host's ability to mount an immune response to a tumor challenge. Conclusions which have been drawn from their mouse studies have suggested that perhaps patients undergoing radical surgery for cancer should have their local lymph nodes left intact in order that their immune response to the tumor is not significantly altered. The

purpose of this study was to determine what effect the regional lymphadenectomy would have on the host ability to develop concomitant immunity to tumor specific transplantation antigens.

Methods Employed:

Two hundred and twenty inbred Balb/c mice were randomly inoculated in the right hind limb with $1x10^5$ viable isologous fibrosarcoma cells from a methylcholanthrene induced tumor. Twenty of these mice served as unoperated controls. The remaining 200 mice were divided into four groups of 50 mice and challenged with $1x10^4$ tumor cells in the left hind limb on days 3, 7, 14, and 21, after initial right limb challenge. Prior to each second tumor challenge, 20 mice underwent right popliteal and inguinal lymphadenectomy and 10 mice underwent a sham operation. The remaining 20 mice served as controls with 10 additional mice serving as controls for the tumor cell dose used for each challenge.

Major Findings:

Significant concomitant tumor immunity was demonstrated by the failure of the second tumor challenge to grow (0-10%) in those mice whose lymphadenectomy was performed on days 7, 14, and 21, as well as in the sham operated and non-lymphadenectomized controls. In contrast, a significant lack of concomitant immunity occurred in those mice whose lymphadenectomy was done on day 3. Forty-two percent of this group grew left and right limb tumors.

Significance to Biomedical Research and Program of the Institute:

This study, along with the previous study in guinea pigs using alymphatic skin pedicles, suggests that although the regional lymph nodes are important for early host recognition for tumor antigens, their later removal does not adversely alter the host ability to mount a tumor specific immune response.

Proposed Course:

Studies will be continued in hopes that further clarification of the role which the local lymph nodes play in the host response to this tumor.

Project Description:

Part VIII. The Immunogenicity of a Murine Renal Adenocarcinoma

Objectives:

Renal adenocarcinoma or hypernephroma is one of the few tumors that have been known to undergo spontaneous regression in humans. In addition, the often benign and bizarre clinical course associated with this tumor suggest that it may be under immunologic control. The absence of a proper animal tumor model system has previously prevented detection of tumor specific antigens associated with any renal cell carcinoma. The purpose of this study was to determine if a renal cell carcinoma arising in an inbred strain of mice, could be shown to contain tumor specific antigen.

Methods Employed:

A renal cell carcinoma which arose in a Balb/c mouse having previously received an injection of a cell-free extract obtained from a human gastric cancer was used for these studies. Once the growth pattern of this tumor had been obtained, animals were immunized by temporary tumor growth and amputation. Following amputation, animals were challenged with a minimal tumor cell dose in order to determine if an immune response existed. Animals appearing to be immune to rechallenge were also challenged with other syngeneic tumors, as well as normal skin to determine the specificity of the immune response.

Major Findings:

The renal adenocarcinoma used in these studies has been found to contain tumor specific transplantation antigens which can protect against rechallenge of syngeneic tumor. There has been no cross-reactivity of this immune response with syngeneic mammary tumor or methylcholanthrene induced tumors. Animals rejecting the renal adenocarcinoma also readily accepted skin grafts from syngeneic animals previously bearing transplants of the renal tumor.

Significance to Biomedical Research and Program of the Institute:

The finding that a renal adenocarcinoma arising in inbred strains of mice does contain tumor-specific transplantation antigens suggests that this may be an excellent model for studies concerning immunotherapy.

Proposed Course:

Further studies will be carried out using this animal model system as a means of immunotherapy alone or in conjunction with chemotherapy.

Project Description:

Part IX. Hormonal and Chemotherapy for Renal Cell Carcinoma

Objectives:

The purpose of this study was to try and find a suitable animal tumor model which simulated human renal adenocarcinoma. Treatment for metastatic renal cell carcinoma in most instances has been disappointing. The poor response rate of advanced renal cell cancer to the more conventional therapeutic modalities has resulted in the utilization of hormonal therapy as an alternative. The experimental evidence supporting hormonal therapy of renal cell cancer is almost solely derived from studies employing the renal tumor induced in the male Syrian golden hamster following prolonged administration of diethylstilbestrol. The hormone dependency of such tumors is further illustrated by the fact that successful transplantation requires the host to receive exogenous estrogens. Testosterone and medroxyprogesterone acetate

have been shown to prevent the induction of the tumor, the latter also inhibiting the growth of the estrogen-dependent transplant. Although clinical trials using these hormones have been initiated, their limited success suggests that the renal cell cancer found in the hamster tumor models may not adequately portray the most common renal tumor in man. The present studies were performed to determine if hormonal or chemotherapy would effect the transplantation of the renal adenocarcinoma which arose spontaneously in a mouse of an inbred strain.

Methods Employed:

The renal adenocarcinoma, designated MKT-CD1, arose spontaneously in a male Balb/Cf/Cd mouse. The tumor has been maintained by serial transplantation in female Balb/C/AnN and was used in its early transplant generation. First and second set full thickness skin grafts exchanged between Balb/Cf/Cd mice and Balb/C/AnN mice had been accepted for over 365 days. All animals received an intramuscular injection of a single cell suspension containing $1x10^4$ viable MKT-CD1 cells, in the right hind limb and were then randomly selected for each experimental group. Hormonal therapy was begun on the fifth day following tumor inoculation and consisted of cortisone, progesterone (Provera), testosterone, and diethylstilbestrol.

Major Findings:

Unlike the golden hamster tumor model, cortisone and progesterone had no effect on the growth of the murine renal adenocarcinoma. Testosterone, however, and diethylstilbestrol both inhibited the renal tumor transplant. This inhibition was greater with the diethylstilbestrol than with the testosterone.

Significance to Biomedical Research and Program of the Institute:

The tumor host system used in these studies is felt to be superior to the hamster model for the following reasons: (1) It is spontaneous, i.e., not induced by exogenous hormones or chemical carcinogens, (2) It resembles the human renal cell tumor both grossly and histologically, (3) The tumor arises in inbred strain of mice allowing transplantation without the fear of tumor inhibition on the basis of histocompatibility antigens, (4) No immunosuppressive or other agents are required for transplantation, (5) It is slow growing and thus similar to the often very slow growing human renal cell cancer, (6) It grows at a reproducible rate, and (7) Studies can be carried out within the same low transplant generation, and (8) A mouse system allows large scale economical studies.

Proposed Course:

This tumor host system will now be used to evaluate a number of chemotherapeutic agents.

Project Description:

Part X. The Production of Bladder Tumors in Inbred Mice and the Study of their Immunologic Properties

Objectives:

Immunologic studies of urinary bladder carcinomas has been hampered by the lack of appropriate experimental models. The canine bladder is sensitive to the carcinogenicity of 2-naphthylamine and 4-aminobiphenyl as well as other compounds regarded as carcinogenic for the bladder in humans. However, the dog is unsuitable for proper immunologic experiments. Bladder carcinomas have been produced in rats and mice using these above compounds and other compounds but only when used in conjunction with a paraffin or cholesterol pellet. The high incidence of bladder carcinomas produced by the paraffin or cholesterol pellets alone adds an additional carcinogen or the possibility of a synergistic effect with the hydrocarbon implantation technique. Recently, N-[4-(5-Nitro-2 furyl)-2 Thiazolyl]formamide or FANFT, a candidate antimicrobial, 5-nitrofuran drug, has been found to produce a nearly 100% incidence of urinary bladder carcinomas when fed to female and male non-inbred rats and mice. Although FANFT has not yet been studied in inbred rats or mice, it appears to be the most suitable compound available to study the immunologic aspects of bladder cancer. It also provides the best experimental model to study the temporal sequence of events in the genesis of urinary bladder carcinomas. In addition, this model may provide the most suitable system yet found to economically study various therapeutic modalities with some assurance that some studies may have some relevance to man. Our studies will include two inbred strains of mice to assure tumor production, since mice strains are noted to differ in their susceptibility to chemical carcinogens. We hope to study the effects of immunosuppression on the production of bladder tumors. In addition, the accuracy of urinary cytology in detecting the early onset of experimental tumors will be studied.

Methods Employed:

Two inbred strains of mice, C57Bl/6N and C3H/HeN will be used during this study. Animals will be fed purina laboratory chow containing the appropriate amount of FANFT. Animals serving as controls will receive their normal diet of purina laboratory chow. Others will be given a diet containing the chemical carcinogen and repeated injection of cytoxan or another immunosuppressive agent. Another group will be given cytoxan alone. All animals will be bled periodically to determine their white blood counts. Urine will be collected from all groups at regular intervals with some of this being put aside for further studies, and a portion of each collection sent for cytologic examinations. Skin grafts will be exchanged between the two different inbred strains in order to determine whether or not effective immunosuppression is being obtained. At regular intervals animals will be autopsied and their bladders examined grossly and microscopically for the evidence of tumors as well as a general autopsy for other tumor production. Tumors once found will be transplanted into syngeneic animals for further immunologic studies.

Major Findings:

There was nearly a 100% incidence of bladder tumors occurring in both strains of animals used. Of interest was the fact that the C3H/HeN strain tumors occurred much earlier than the C57Bl/6N strain. Urinary cytology was not accurate in being able to detect early cancers, however, the occurrence of microscopic hematuria correlated well with the development of bladder tumors. As the tumors were found at the various intervals they were transplanted to new-born syngeneic mice. It is of interest that not all of these tumors grew, indicating perhaps that some may be benign in nature. Preliminary immunologic studies suggest that the bladder tumors contain tumor specific transplantation antigens which are distinct for each tumor. Animals immunosuppressed with cytoxan had the same incidence of bladder tumors as those not immunosuppressed. A lymphocytic infiltrate was noted in the walls of those bladders with tumors suggesting an immunologic response to the new tumor antigens. Animals receiving cytoxan had no appreciable lymphocytic infiltrate and yet, the incidence of tumors was the same. These findings would suggest that if the lymphocytic response is directed to tumor specific antigens it has little effect on the initiation of these bladder tumors.

Significance to Biomedical Research and Program of the Institute:

It is hopeful that this animal model system will produce important information regarding the immunologic aspects of cancer as well as information regarding the temporal sequence of events in the genesis of urinary bladder cancers.

Proposed Course:

Further studies are being carried out to better define the immunologic properties of these bladder cancers.

Project Description:

Part XI. Tissue Culture Studies of Human Prostatic Tissues

Objectives:

The major objective of this project was to establish an *in vitro* cell line derived from normal and adenomatous and neoplastic prostatic epithelium.

Methods Employed:

Thus far, attempts have been made to grow only adenomatous prostatic tissue. These tissues have been grown out of mixed plant cultures using standard techniques.

Major Findings:

Three cell lines have been established. Two lines appear fibroblastic but the others appear epithelial in culture. The epithelial cell line has been carried through more than 100 cell line passages. Electron microscopic studies indicated that these cells are epithelial in nature. The cells contain prostatic acid phosphatase. The cells produced solid tumors when injected into the cheek pouch of the immunosuppressed hamsters. The cells grow in spinner cultures. In a sense, then, the cells have undergone "spontaneous transformation."

Also, while these cells were being grown in high concentration of zinc, they lost contact inhibition. Discret-foci were observed by electron microscopy with virus-like particles. Previously, these cells had grown as monolayer cultures. These observations are under intensive investigation at the present time.

Significance to Biomedical Research and Program of the Institute:

We feel that it is important to establish a cell line derived from prostatic epithelium in tissue culture so that some basic aspects of prostatic cellular physiology can be studied.

Proposed Course:

Work will continue developing epithelial cell lines derived from human adenomatous prostate glands. Also, attempts will be made to grow the neoplastic elements of human carcinoma of the prostate.

Objective and Approach of the National Cancer Program:

Objective 2: To modify individuals, e.g., by vaccination to decrease the likelihood of cancer development both in the current generation and in subsequent offspring.

Approach 1: Reduce development of cancer by altering immunological capability of individuals.

Publications:

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Serial No. NCI-3820
1. Surgery Branch
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cellular Immunity to Tumor Specific Transplantation Antigens

Previous Serial Number: Same

Principal Investigators: A.S. Ketcham, M.D., and A.M. Cohen, M.D.

Other Investigators: J.F. Burdick, M.D., S.A. Wells, M.D. and R.C. Millar, M.D.

Cooperating Units: None

Man Years:

Total:	3-1/2
Professional:	1-1/2
Other:	2

Project Description:

Part I. Studies of Cellular Immunity to Tumor Specific Transplantation Antigens in a Guinea Pig Tumor Host System

Part II. Response to Skeletal and Soft Tissue Sarcomas as Studied In Vitro with an Immune Cell Mediated Cytotoxic Assay

Part III. A Cytotoxic Assay for Humoral Immunity

Project Description:

Part I. Studies of Cellular Immunity to Tumor Specific Transplantation Antigens in a Guinea Pig Tumor Host System

Objectives:

To study in vitro the splenic lymphocyte response to concomitant tumor growth in a syngeneic Strain 2 guinea pig tumor host system. To study the possibility of the development of a humoral efferent enhancing antibody.

Methods Employed:

Two noncrossreacting methylcholanthrene induced sarcomas in Strain 2 guinea pigs have been studied. An assay for in vitro lymphocyte mediated cytotoxicity was developed. This technique involved the labeling of tissue culture target cells with I^{125} labeled iododeoxyuridine, a DNA label. After the addition of immune lymphocytes to the tissue culture the loss of radioactivity from the monolayer culture indicated the degree of lymphocyte cytotoxicity.

Major Findings:

The reliability of this in vitro assay of cell mediated immunity was confirmed in a number of experiments. Animals immunized to the methylcholanthrene induced tumors developed immune lymphocytes which were capable of lysing I^{125} iododeoxyuridine labeled target cells. The addition of serum from animals with progressively growing tumors abrogated this lymphocyte mediated cytotoxicity indicating the presence of a "blocking factor".

Significance to Biomedical Research and Program of the Institute:

The methodology of labeling target cells with I^{125} iododeoxyuridine represents an advance in cell mediated immunity. This label is unique in that there is minimal leak from live cells and upon immune cytolysis the label is not reutilized by bystander cells. By using a gamma emitter, the label is much easier to count than methods which use other isotopes. The presence of a "blocking factor" in the serum of animals with progressively growing tumors which abrogates the lymphocyte mediated cytotoxicity is in agreement with the Hellstroms' findings. Fractionation of this antibody should yield further information about its properties.

Proposed Course:

Attempts will be made to separate and characterize the "blocking factor". The I^{125} iododeoxyuridine label is also being used as a measurement of lymphocyte transformation in recognition of tumor-specific antigens. This cell mediated immune system is also being used to detect cellular immunity in human tumor systems.

Project Description:

Part II. Response to Skeletal and Soft Tissue Sarcomas as Studied In Vitro with an Immune Cell Mediated Cytotoxic Assay

Objectives:

The presence and degree of human cellular immunity developing to melanomas and sarcomas and the presence or absence of central and efferent enhancing antibody is to be studied. The presence of crossreactive tumor specific

antigens will be approached with allogeneic cytotoxic lymphocytes.

Methods Employed:

Over the past several years multiple tumor lines and associated skin fibroblasts or muscle lines have been obtained on patients with melanomas and sarcomas by Donald L. Morton, M.D., in the Surgery Branch of the National Cancer Institute. These lines have been used in a study of allogeneic cellular immunity to tumor specific transplantation antigens. Peripheral blood lymphocytes are obtained from heparinized blood by a carboneal iron methylcellulose extraction technique which produces 99% pure lymphocytes. The labeling of target cells with I^{125} iododeoxyuridine allows for accurate quantitation of these cellular immune responses. Cytotoxicity was studied using blood from patients who were seen in the surgical clinic for routine follow-up visits. Many of these patients have been cured of their disease. Some patients had developed metastatic disease and changes in their cellular immunity during this period of time and the question of presence of central enhancing or efferent enhancing antibody was investigated.

Major Findings:

It has been shown that patients with skeletal and soft tissue sarcomas have lymphocytes which demonstrate immune cytotoxicity against a sarcoma line growing tissue culture but did not demonstrate cytotoxicity against normal skin grown from the same patient carrying the sarcoma. This lymphocyte mediated cytotoxicity against sarcoma tumor cells could be blocked by preincubating the tumor target cells with serum from patients with progressively growing tumors. Preoperatively most patients with skeletal and soft tissue sarcomas seem to have this factor in their serum which blocks cell mediated immunity to the sarcoma tissue culture cells. Following curative resection of the sarcomas these patients no longer demonstrated this factor in their serum. This is in agreement with the finding of the Hellstroms; that there is some "blocking factor" which prevents *in vivo* immune lysis of tumor cells by lymphocytes.

Significance to Biomedical Research and Program of the Institute:

It is now well established that skeletal and soft tissue sarcomas carry tumor cell surface antigens which are common between several skeletal and soft tissue sarcomas from different patients. This common antigen has been demonstrated not only by cell mediated immunity as reported presently but also by complement fixation, immunofluorescence and humoral cytotoxicity. The fact that patients with progressively growing tumors have a factor in their serum which abrogates the cell mediated cytotoxicity is in agreement with the Hellstroms' finding of an enhancing or blocking factor in the serum of patients with tumors. Current work is involved with the development of cell mediated immunity to other tumor systems.

Proposed Course:

Attempts are being made to correlate cell mediated immunity and the presence of "blocking factor" with the patient's clinical course. Also, efforts will be made to modify the reactivity of autologous lymphocytes to make them more effective against ablating the rest of the growing sarcoma tumor cells in vivo.

Project Description:

Part III. A Cytotoxic Assay for Humoral Immunity

Objectives:

To study in vitro the efficacy of an assay for humoral cytotoxicity.

Methods Employed:

Using various animal allograft and tumor isograft systems, a technique of studying humoral cytotoxicity has been investigated. This involves labeling target cells with I^{125} iododeoxyuridine. Cytotoxic antibody and complement are then added to the system and after incubation overnight, the loss of target cells from tissue culture is monitored by the release of I^{125}.

Major Findings:

The use of I^{125} labeled target cells has been applied successfully to the study of humoral cytotoxicity. This test is much less subjective than previously described assays which depend on visual counting of cells present.

Significance to Biomedical Research and Program of the Institute:

A simple, if possible, automated technique of in vitro humoral cytotoxicity is necessary for screening large numbers of sera from patients with various malignancies. The I^{125} labeled target cell technique is relatively simple and could be adopted to such a protocol.

Proposed Course:

Further animal and human studies are being carried out with the I^{125} iododeoxyuridine labeled target cells. Human tumor systems are being utilized at present to detect the presence of humoral mediated immunity in human tumor systems.

Objective and Approach of the National Cancer Program:

Objective 3:

To prevent conversions of cells to those capable of forming cancers (i.e., block or interfere with the proximate step, or steps, involved in conversion to capable of forming cancers).

Approach 5:

Study of cell surface and cell membranes.

Publications:

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Morton, D.L., and Wells, S.A.: Immunobiology of neoplastic disease. In Davis-Christophers Text Book of Surgery (10th Ed., 1972), W.B. Saunders Co., Philadelphia, (in press 1971).

Serial No. NCI-3808
1. Surgery Branch
2. Tumor Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of Host Factors Associated with Implantation and Growth of Cancer

Previous Serial Number: Same

Principal Investigator: Donald L. Morton, M.D.

Other Investigators: F.R. Eilber, M.D., W.C. Wood, M.D., B.K. Gray, M.D., and J.T. Mehigan, M.D.

Cooperating Units: Laboratory of Immunology, NIAID

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Part I. Immunologic and Virus Studies with Human Neoplasms

Part II. Cytotoxic Activity in the Serum of Sarcoma and Melanoma Patients

Part III. Prognostic Significance of Cutaneous Anergy in Patients with Solid Neoplasms

Part IV. Immunotherapy in Patients with Melanomas and Sarcomas

Project Description:

Part I. Immunologic and Virus Studies with Human Neoplasms

Objectives:

Tumor-specific antigens have now been demonstrated in several viral and carcinogen-induced animal neoplasms. It is logical to assume that human neoplasms contain cancer-specific antigens similar to those found in these animal tumors, but there is presently no unequivocal evidence for the existence of such antigens. An initial step in improving the existence

of cancer-specific antigens in human neoplasms would be the demonstration of antibodies in the serum of cancer patients which react specifically with autologous tumor tissue. The objectives of this study are to determine whether antitumor antibodies can be demonstrated by immunological techniques in the serum of cancer patients and to determine the antigenic specificity of the tumor antigens from the various types of human neoplasms.

Methods Employed:

Specimens of tumor were obtained from patients with melanomas and sarcomas. Tumor imprints were made on glass slides which were then stored in liquid nitrogen until used in immunofluorescent studies. The remainder of the tumor specimens were preserved by slow freezing in DMSO and stored in liquid nitrogen also. Normal tissues from the same organ and other normal tissues from the same patient were similarly treated. In addition, the patient's serum and serum from other patients with the same neoplasm, and normal sera were obtained and tested by the direct and indirect immunofluroescent techniques against the acetone-fixed tumor imprints. Similar sera were tested by the complement fixation against extracts prepared from the tumor tissue culture cells.

Major Findings:

Immunologic studies with sarcomas: Immunofluroescent studies revealed a high incidence of antibodies to osteosarcomas in the sera of patients with this disease and their close associates which react with a common antigen in osteosarcomas. Serum randomly selected from six patients with osteosarcoma, 24 immediate family members of the osteosarcoma patients, four close associates of one osteosarcoma patient, and 24 normal blood bank donors were tested by immunofluorescent techniques for antibodies against six different osteosarcomas. Each patient's serum was found to react not only to his hown osteosarcoma, but also in 80% of tests with the tumors of other patients. A high incidence of immunofluorescence antibodies was also demonstrated in tests with serum from patients of the immediate family and the serum from close associates of one patient. The tests of serum from normal blood bank donors revealed a significantly lower incidence of this antibody than in the serums of patients, their relatives, or close associates. The results observed with the blocking reaction were similar to those previously reported using the indirect immunofluorescent technique. The unlabeled serum from the osteosarcoma patients as well as the relatives of osteosarcoma patients blocked the reaction of fluorescein labeled globulin of patients against their own osteosarcoma cells as well as against the osteosarcoma cells of two other patients. This indicates that all these serums contain antibodies against the same or closely related osteosarcoma antigens. The specificity of these reactions were studied by testing the fluorescein labeled globulins of osteosarcoma patients against other human neoplasms. These sera frequently reacted with sarcomas of other types, particularly liposarcomas, fibrosarcomas, and myosarcomas. The reactions were also seen with two of four melanomas tested, but not with adenocarcinomas of the breast and colon.

The increased incidence of antibodies to osteosarcoma in serum from immediate family members and close associates of osteosarcoma patients suggests the association of an infectious agent with this neoplasm. This agent appears to be capable of infecting close associates of these patients in whom it elicits the formation of antibodies reacting with an antigen in osteosarcomas. The cross-reactivity between sera from one osteosarcoma patient and his family members with another patient with osteosarcoma suggests that most osterosarcomas contain the same tumor antigen. The cross-reactivity of the serums from osteosarcomas with some soft tissue sarcoma suggests that these neoplasms may also be associated with an antigenically similar agent.

A sarcoma specific antigen has been found in tissue culture cells derived from several different histologic types of human sarcomas. This sarcoma-specific antigen is not present on normal fibroblasts of sarcoma patients in non-sarcomatous malignancies, normal human organs, or fetal organs. The sarcoma-specific antigen is cell-associated but may be solubilized from tissue culture cells of human sarcomas by a variety of techniques. This soluble antigen is quite stable and has been partially purified by chromatography in polyacrylamide gel electrophoresis. This antigen appears to be a protein since it is partially inactivated by trypsin and fully inactivated by pronase. The partially purified antigen reacts well with specific sarcoma antibodies in human sera by complement fixation and has been found to produce a delayed cutaneous hypersensitivity reactions in patients with sarcoma, but not in patients with other types of malignancies.

The presence of a filterable agent in human sarcomas was directly demonstrated by the finding that cell-free extracts both from sarcoma tumor tissue and concentrated media from the tissue culture cells of human sarcomas were capable of infecting normal human embryonic cells in tissue culture where they induced the formation of a new sarcoma-specific antigen so that these antigenically transformed normal cells now reacted with the serum from sarcoma patients, whereas the normal uninfected cells have not.

Furthermore, absorption of the sarcoma test sera with the sarcoma tissue culture cells removed all the activity against the original sarcoma culture as well as against the transferred antigen. This indicates that the filterable agents inducing the new antigen was the same as the agent inducing the antigen in the original sarcoma.

A complement fixation test has been developed for study of human tumor antigens. This assay utilizes selected fresh human serum as a complement source. It provides a quantitative, sensitive and reproducible technique for the study of the human tumor antigens and antibodies. With this test, sarcoma-specific antibodies have been found in 92% of the sera of patients with skeletal and soft tissue sarcomas with a mean titer of 1/98. The incidence of antibody in the serum of normal blood bank donors is 21% with a mean titer of 1/17. A larger number of patients with other types of malignancies have been tested and found not to contain either a higher incidence or titer of

antibody than that found in normal blood bank donors. The titer of this antisarcoma antibody shows an excellent correlation with the clinical course of the patient's malignant disease. Antibody titers rise following surgery and remain elevated in those patients who remain free of tumor. With recurrent disease, however, the titers fall. Thus, there is a considerable correlation between the level of antisarcoma antibody and the course of malignant disease in a given patient.

Immunologic studies with melanoma: Using the indirect immunofluorescent technique, thus far, antimelanoma antibodies have been demonstrated in the sera of 16 patients tested against their own melanoma. A considerable correlation has been found between the clinical status of melanoma and the presence of antimelanoma. The titer of antibody against autologous tumor tissue has been significantly higher in patients with localized melanoma than in those with widespread disease. Also, in cross-reacting tests against sera of eight different homologous melanomas, the incidence of positive tests was 75% in patients with localized disease and only 50% in patients with advanced tumor.

Complement fixation studies demonstrated specific antimelanoma antibody in the sera of melanoma patients against a common melanoma antigen derived from tissue cultures of human melanoma. This antigen is not found in normal fibroblasts, other malignancies, normal adult or fetal human organs in tissue culture. Specific antimelanoma antibody was found in higher incidence and in higher titer in patients with localized melanoma and in patients undergoing spontaneous regression of melanoma than in patients with advanced disease. Furthermore, there was a progressive decline in antimelanoma antibody titers of patients with melanoma as their disease became progressive.

Significance to Biomedical Research and Program of the Institute:

These studies demonstrate a common and tumor-specific antigen in human melanomas and sarcomas. If these specific antigens can be isolated and purified, they should be useful as immunogens in active immunotherapy as an adjunct to definitive surgical treatment of these patients. The studies which demonstrate the presence of an infectious agent in some human sarcomas has considerable importance to the problem of the virus etiology of human cancer. It is possible that purified sarcoma antigen may be useful as a vaccine to prevent the development of these malignancies in susceptible population.

Proposed Course:

Studies designed to purify and further characterize the tumor-specific antigens whose presence are demonstrated by these studies are in progress.

Project Description:

Part II. Cytotoxic Activity in the Serum of Sarcoma and Melanoma Patients

Objectives:

To develop a cytotoxicity test to assay the incidence of cytotoxicity in the serum of patients with these tumors and evaluate the specificity of this assay.

Methods Employed:

A microcytotoxicity test was developed for this assay. Human malignant cells grown in monolayers were harvested and implanted in microtest tissue-culture plates. Heat-inactivated test sera was added and after a one-hour incubation, the serum was removed and complement added. After further incubation, the wells were examined by phase-microscopy and viable cells counted.

Major Findings:

Serum from each of four patients from whose sarcomas the tissue-culture cell lines were derived were tested for autologous cytotoxicity. All four of the sera demonstrated cytotoxicity against their own tumor cells. One of the established osteogenic sarcoma cell lines was selected as the target cell for screening a large number of sera. Seventy percent of the sera from patients with skeletal and soft tissue sarcomas were found to demonstrate significant cytotoxicity. By contrast, only 8% of the blood bank serum exhibited cytotoxicity, and less than 10% of sera from 82 patients with other malignant tumors proved cytotoxic. These data suggest the presence of a common cell surface antigen in human sarcomas which renders the cell vulnerable to immune cytolysis by cytotoxic antibody.

Cytotoxic antibodies were also demonstrated in the sera of melanoma patients against their own autologous cells in culture. Many of these sera were also cytotoxic to the cell lines derived from other patients' melanomas, but the extent of cross-reactivity varied between cell lines from 75% to 25%.

Significance to Biomedical Research and Program of the Institute:

These studies indicate that the tumor-specific antibodies demonstrated in the sera of patients with sarcomas and melanomas are directed against cell surface antigens or intracellular components. The complement dependent cytotoxic activity in the serum of these patients strongly suggests that they have antibody directed against antigens expressed on the cell surface. This provides a convenient method of following immune response of these patients after treatment with tumor vaccines or other agents which are designed to alter the immune response.

Proposed Course:

This assay system will be used to monitor the effects of immunotherapy in patients with melanomas and sarcomas.

Project Description:

Part III. Prognostic Significance of Cutaneous Anergy in Patients with Solid Neoplasms

Objectives:

To study the capacity of preoperative patients with localized, solid neoplasms to display delayed cutaneous hypersensitivity to a variety of skin test antigens and relate this immunological reactivity to the subsequent course of the patient's malignant disease.

Methods Employed:

Patients ranging in age from 14 to 70 years who had a variety of clinically operable solid tumors were studied. Delayed cutaneous hypersensitivity (DCH) to dinitrochlorobenzene was studied, using the technique of Brown, et. al. This involves sensitization with 2000 µg DNCB and challenge two weeks later with 50 µg DNCB. A battery of seven common skin test antigens were also used for DH testing.

Major Findings:

Patients with benign disease, long-term survivors following treatment of malignant neoplasms or with spontaenous regression all had normal DH to DNCB. By contrast, only 64% of 110 preoperative cancer patients manifested DH to DNCB. Ninety percent (63/70) of the operable patients free of disease six months after treatment were positive to DNCB. However, only 10% (7/70) of those patients who were either inoperable or who developed metastases within six months after surgery reacted to DNCB preoperatively. This was significantly different from the 90% reactivity exhibited by operable patients free of disease for this interval. Also, seven patients with malignant melanoma or skeletal or soft tissue sarcomas, who reacted to DNCB preoperatively, subsequently developed widespread metastases and retained their ability to react to DNCB until death.

The incidence of reactivity to one or more antigens of the preoperative group (39%) was significantly different from the control group (90%). Those patients with benign diseases (80%), long-term survivors (60%), and patients with spontaneous regression (50%) reacted to one or more of the antigens. Of those operable patients free of disease for six months, 60% were positive preoperatively, whereas those who were inoperable or who developed early metastases, only 20% reacted to the antigens.

It was thus found that DNCB reactivity more closely predicted the subsequent course of neoplastic disease in a given patient than the common antigens, since only 3% of patients free of disease were non-reactive to DNCB, but 40% of thos patients who were free of disease were negative to all common skin test antigens.

Significance to Biomedical Research and Program of the Institute:

Increasing evidence that human malignancies contain tumor-specific antigens provide the firm theoretical basis for the observed relationships between immunologic competence and the regression of human neoplastic disease reported in this study. These observations also provide a more rational basis for future immunotherapy to control malignant disease. Hopefully, the evaluation of cell-mediated immunologic reactivity will be useful in planning this therapy: active immunization in immunologically competent hosts and passive immunotherapy in an anergic host would appear to have the greatest hope of success.

Proposed Course:

DNCB reactivity is now assayed using a quantitative method that more accurately determines the immunologic status of cancer patients. These studies will be used to monitor the effect of adjunctive therapies (chemotherapy and immunotherapy).

Project Description:

Part IV. Immunotherapy in Patients with Melanomas and Sarcomas

Objectives:

To study the effects of immunization with tumor antigens and immunological adjuvants on the course of disease in patients with inoperable melanomas and sarcomas. To correlate these effects with antitumor antibody production as assayed by complement fixation and cytotoxicity tests.

Methods Employed:

Patients with sarcomas and melanomas that were clinically inoperable were studied. Twelve patients with inoperable sarcomas were immunized with autologous tumor antigen and BCG. Immunization was repeated at intervals that depended on the patient's general condition and response to therapy. Eighteen melanoma patients with multiple cutaneous metastases had injection of BCG directly into their metastatic lesions. Additional intradermal injections were made in the regions proximal to major lymph node areas at intervals after injection of the subcutaneous nodules.

Major Findings:

When serum cytotoxic antibody was assayed by a microcytotoxicity technique, nine of 12 sarcoma patients showed an increase of cytotoxic antibody after immunization with a combination of sarcoma antigen and BCG when the serum was tested against a homologous sarcoma tissue culture cell line. The tumor specificity of this response was apparent in seven patients by the demonstration that these sera were not cytotoxic against normal control fibroblasts.

Tests of post-immunization sera of two patients against autologous sarcoma tissue culture cells showed an increase in cytotoxic activity which was not evident in concurrent tests against autologous fibroblasts. Three patients showed no response to autologous cellular vaccination, and no demonstrable cytotoxic antibody throughout their clinical course. The sera of 7/9 patients demonstrated a rise in antisarcoma antibody after immunization when assayed by complement fixation. Skin tests were also performed, utilizing irradiated autologous tumor cells. Positive delayed cutaneous hypersensitivity reactions to autologous tumor cells which were not present prior to immunization became positive in 6/9 patients. These results emphasize that even in patients with metastatic sarcoma, it is possible to augment both the humoral and cellular immune response to the malignancy. Although no alteration of the clinical course was noted in any patient, two patients have had clinically tumor-free intervals after immunization of 12 to 18 months, respectively, during which their cytotoxic antibody titers have remained high.

In patients with melanoma who were tested with BCG, DNCB positive patients had at least a four-fold rise in the titer of antimelanoma antibody as determined by complement fixation as well as regression of melanoma nodules directly inoculated with BCG. Of 18 patients treated thus far, five have shown some response to therapy. One had complete remission of injected and uninjected nodules and remains free of disease at three years following immunotherapy, and two others have had dramatic regression of multiple metastatic nodules, some of which were not injected with BCG, and several nodules remaining in each patient have been dormant over a two-year interval.

Significance to Biomedical Research and Program of the Institute:

These preliminary studies demonstrate that both specific and non-specific stimulation of immune systems in patients with sarcomas and melanomas increase serum and cellular immune reactivity against the tumor. Failure to obtain clinical remissions in most patients must be viewed in the context of the disease status of the patients treated. All had diffuse metastases in the presence of which, even with the most efficacious chemotherapy regimen, only minimum symptomatic paliation intervals would have been achieved. A more realistic goal for immunotherapy is as an adjuvant to definitive surgical treatment for the irradication of the remaining microscopic foci of tumor. These results suggest that such combined therapies have a reasonable chance of success.

Proposed Course:

Protocols are now being initiated that utilize specific and non-specific immune stimulants in patients who have had resection of all clinically apparent tumor, but in whom previous clinical experience indicates recurrent tumor will soon be manifest.

Objective and Approach of the National Cancer Program:

Objective 6. To cure as many patients as possible and to maintain maximum control of the cancerous process in patients not cured.

Approach 1. Enhance the host's ability to eliminate or prevent further development of cancer.

Publications:

Morton, D.L., and Eilber, F.R.: Prognostic significance of cutaneous anergy for the surgical treatment of solic neoplasms. In Hall, T.E. and Foye, L. (Eds): Prognostic Factors in Human Cancer. National Cancer Institute, Monograph No. 34, 103-108, 1971.

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PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1972

Project Title: Tissue and Organ Transplantation

Previous Serial Number: Same

Principal Investigators: D.L. Morton, M.D., W.L. Joseph, M.D., and P.B. Chretien, M.D.

Other Investigators: A.S. Ketcham, M.D. and F.R. Eilber, M.D.

Cooperating Units: Laboratory of Immunology, NIAID

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Part I. Physiologic and Morphologic Alterations in the Transplanted Baboon Lung

Part II. Influence of Ischemia and Hyperthermia on the Function of the Transplanted Lung

Part III. Quantitation of Immunosuppressive Therapy by Dinitrochlorobenzene Contact Sensitivity

Part IV. The Effect of Phytohemagglutinin on In Vivo Immune Responses in the Guinea Pig

Project Description:

Part I. Physiologic and Morphologic Alterations in the Transplanted Baboon Lung

Objectives:

To study a physiologic and cytoarchitectural changes over an extended period of time in the lungs that provided immediate and total respiratory function after transplantation.

Methods Employed:

Previous studies in this laboratory have demonstrated superiority of the baboon for studies in lung transplantation. A technique of left lung autotransplantation and simultaneous ligation of the contralateral pulmonary artery has been developed. This study reviews the experience with 30 baboons who have had transplanted lungs functioning for a long period of time. Arterial oxygen partial pressure in the pulmonary artery have been measured preoperatively and at intervals postoperatively. Percutaneous lung biopsies have been performed at the same time. Various survivors were killed beginning at three months after transplantation and up to one year. Postmortum studies were performed on these baboons, and gross and microscopic examination of the lungs and heart were carried out.

Major Findings:

After left lung autotransplantation and simultaneous ligation of the contralateral pulmonary artery, there is a temporary fall in arterial pO_2-mean of 64.4 ml of mercury, although 12 of 30 baboons showed a transit elevation in pulmonary artery pressure with a mean increase of greater than 10 ml of mercury. X-rays and lung biopsies done in this period show changes of congestion characterized by diffuse proteinaceous alvelar exudate as well as interstitial edema, intravascular congestion and areas of local alvelar collapse. By two weeks, resolution of the congestion is seen roentgenographically and pulmonary artery pressure returned to preoperative levels. However, the arterial pO_2 remains somewhat depressed. Biopsy shows continued intravascular congestion, interstitial edema, and some alvelar exudate. At one month, hemodynamic studies reveal little alteration and there is almost complete resolution of alvelar exudate and edema but persistent of mild intravascular congestion. After one month, blood gas values and right heart pressure studies are stable. There is a mean elevation of pulmonary artery pressure of 3-4 ml of mercury that persists during a one-year follow-up. There is also persistent mild interstitial congestion, diffuse interstitial leukocytes and macrophage infiltration, and patchy areas of bronchopneumonia. Arteriography showed dilitation of the pulmonary vasculature. By nine months, the lung showed fibrous thickening and hypertrophy of alveloi septic and fibrine disposition in extravascular spaces. Postmortum examination on baboons killed after six months show mild hypertrophy of the right ventricular walls with dilitation of the internal vein pulmonary trunk and left pulmonary arterial tree.

Significance to Biomedical Research and Program of the Institute:

Relatively little is known concerning changes in pulmonary cytoarchitecture over an extended period of time in the transplanted lung forced to provide immediate and total respiratory function. These alterations are particularly important since, in human lung allografting, the major source of respiratory support will be the transplanted lung. In addition, certain clinical situations, such as pulmonary hypertension, will require the allografted lung to

accept an increased proportion of the heart output. This study supports previous clinical and experimental findings that a transplanted lung can provide adequate respiratory function immediately after allografting. In addition, it demonstrates that long-term functional adequacy is possible with apparently minimum deterioration in morphological integrity up to a period of one year after allografting.

Proposed Course:

Continued studies using this experimental model will provide data vital for the planning of lung transplantation in humans.

Project Description:

Part II. Influence of Ischemia and Hyperthermia on the Function of the Transplanted Lung

Objectives:

To determine the duration of ischemia at various temperatures compatible with immediate function of a transplanted lung.

Methods Employed:

Left lung autotransplantation and immediate contralateral pulmonary artery ligation was performed in baboons. The transplanted lung was subject to various periods of ischemia and hyperthermia preservation prior to graft. All of the baboons were studied preoperatively and postoperatively by serial chest X-rays, arterial blood oxygen determinations, right heart catherization, and pressure studies. Periodic lung biopsies were obtained in all postoperative survivors, with gross and microscopic examinations performed at the time of death.

Major Findings:

Forty-three baboons were studied following left lung autotransplantation and immediate contralateral pulmonary artery ligation. Immediate and total respiratory function could be obtained after four hours of normal thermic ischemia with the lung inflated. An additional hour of ischemia was tolerated by adding continuous ventilation and external surface cooling at 10°C. Hyperthermic perfusion allowed only three hours of ischemia with consistent survival and function of the transplant. In those transplants subjected to ischemia beyond the limits, the immediate cause of death was pulmonary edema and was associated with a precipitous fall in respiratory function and the development of intolerable pulmonary artery pressures. The degree of parenchymal damage and reversibility appears to be dependent on the amount of anoxic damage to the grafted lung.

Significance to Biomedical Research and Program of the Institute:

Cadavers appear to be the only practical source for clinical lung allotransplantation. As a result, a period of ischemia for the lung graft will be unavoidable clinically, and, therefore, adequate techniques of organ preservation will be necessary to maintain pulmonary viability. Most studies of lung preservation in experimental animals have been performed with the contralateral lung intact. However, the ultimate test of a preservation technique for a transplanted lung is its ability to provide immediate and total respiratory function which will support the life of the individual. The present study presents an experimental model which closely approximates the clinical feature and problems involved in lung transplantation in humans.

Proposed Course:

Continued experimentation with various perfusates in combination with alterations in ventilation and variation in oxygen content of the scintillating gases will be explored in an attempt to achieve greater periods of ischemia. The data obtained will be incorporated in clinical lung transplantation and protocols.

Project Description:

Part III. Quantitation of Immunosuppressive Therapy by Dinitrochlorobenzene Contact Sensitivity

Objectives:

This study was undertaken to determine whether the ability to manifest late cutaneous hypersensitivity (DH) to dinitrochlorobenzene (DNCB) could be used to measure immunosuppressive effectiveness of drug therapy in an allograft transplantation system.

Methods Employed:

Skin allografts were transplanted in randomly selected mongrel dogs. Prior to transplantation, each was sensitized to topical DNCB. The animals were maintained on a regimen of Prednisone and Immuran postoperatively. Correlations were made between DH to DNCB, immunosuppressive drug dose, and organ transplant survival data.

Major Findings:

With maximum immunosuppression regimen, the skin grafts survived for over 21 days. During this time, the animals were DNCB negative. DNCB positivity returned, however, when the immunosuppressive regimen dose was reduced. In animals with allografts that received no immunosuppression, there was persistent DH to DNCB.

Significance to Biomedical Research and Program of the Institute:

Although most of the technical aspects of organ transplantation have been mastered, the precise control of rejection still has not been achieved. The early diagnosis of rejection is essential in clinical organ transplantation so that appropriate immunosuppressant therapy can be instituted before irreproachable damage occurs to the transplanted organ. Nevertheless, one of the major problems in organ transplantation is quantitating immunosuppressive therapy on an individual basis, since the major complication of drug therapy is related to the use of high dosages of these drugs over a prolonged period of time. The present experiment indicates that the commonly used combination of steroids and Immuran at effective levels also effects DH to DNCB. It showed that anergy to DNCB is a convenient method of monitoring the immunosuppressive therapy and return of DNCB reactivity correlates with decrease in drug dosage to ineffective levels.

Proposed Course:

Further investigations with this experimental model system will be pursued with the goal of eventual adaptation of this system for following the course of immunotherapy regimens in human renal allograft recipients.

Project Description:

Part IV. The Effect of Phytohemagglutinin on In Vivo Immune Responses in the Guinea Pig

Objectives:

This is part of a systematic investigation of the effects of phytohemagglutinin (PHA) on immune responses in vivo. Previous studies have assessed the effects of PHA on immunosuppressive regimens in dogs. In the present study, the effect of PHA on the immune response to bovine gamma globulin (BCG) tuberculin (DCG) and skin allografts in guinea pigs was determined.

Methods Employed:

Hartley strain guinea pigs were used to study the effects of PHA on the immune responses to BCG and DCG. The animals were immunized with a subcutaneous injection in the nuchal region with a 2% solution of BCG containing hear-killed Microbacterium Bovis (BCG) at a concentration of 5 mg per ml. The animals were bled by cardiac puncture at 1, 2, and 3 weeks after immunization, and serum antibody titer to BCG assayed by the tanned red cell hemogluttination technique. Titer was expressed as the reciprocal of the highest dilution of serum showing 4+ agglutination macroscopically. A portion of the animals immunized as above were challenged with one microgram of tuberculin-D at 2, 3, 5, 7, and 9 weeks after immunization. The mean diameter of erythema was measured 24 hours after this challenge. Inbred guinea pigs, Strain NIH 2 and 13, were used in the study of skin allograft survival.

Full thickness circular skin grafts, 3.5 cm in diameter, were exchanged orthotopically between Strains 2 and 13 guinea pig pairs. On day 8, 10, 12, and 14, the eliptical biopsies were taken at the periphery of the graft. The allograft survival time was determined by microscopic examination of histologic sections prepared from the biopsies. The criteria for rejection was a uniform mononuclear cell infiltrate in the graft dermis. All of the experimental animals received 1 ml of phytohemagglutinin-P (PHA) intraperitoneally daily, commencing two days prior to immunization, challenge, or skin grafting.

Major Findings:

In comparison with the titers in untreated animals, there was a significant delay in the rise in anti-BGG antibody titer in the PHA-treated guinea pig. Two weeks after immunization, the mean $\log_2$ titer of the untreated guinea pigs was 10, while the titer in PHA-treated animals was 5.5 ($P < .005$). Delayed cutaneous hypersensitivity to intradermal tuberculin challenge was completely suppressed in the PHA-treated animals at the second and third week after immunization. After the three week challenge, PHA was discontinued in the test animals. They were rechallenged again at five weeks, and all developed positive skin reactions. PHA was administered to these animals again for three days commencing two weeks prior to the challenge at seven weeks. None developed a skin reaction to this challenge. Two weeks later, when rechallenged without concomitant PHA administration, all animals again developed positive skin reactions.

Significance to Biomedical Research and Program of the Institute:

Since the first demonstration of an immunosuppressive action of PHA in 1965, a number of investigators have assessed the effect of this agent on in vivo immune responses to various antigens. Unfortunately, these studies have thus far yielded conflicting results. In individual experiments, PHA both suppressed and enhanced humoral antibody formation. It prolonged skin allograft survival in some experiments, but not in others. Its effect on delayed cutaneous hypersensitivity and as an adjunct to immunosuppressive drug regimens have similarly varied. The incompletely settled question of the effect of PHA on immune responses may be a particularly important one, since PHA has been administered to humans on many occasions without adverse effects and therefore, could be possibly used clinically as an immunosuppressant. Previous assays of the effect of PHA on immunity were conducted in mice, rats, rabbits, and dogs. Because of the interest in the effect of PHA on cellular immune responses, and the superiority of the guinea pigs for such investigations, this animal was chosen for a broad study of the immunosuppressive action of PHA. This first reported assay of the effect of PHA on immune responses in the guinea pig, and first assay of the effect of PHA on a spectrum of immune responses in any test animal, corroborates previous studies in this laboratory and by other investigators which show that PHA is a potent immunosuppressive agent.

Proposed Course:

Publication of data. Future investigations will be explored in the mechanism of suppression of immunity by PHA.

Objective and Approach of the National Cancer Program:

Objective 7: To restore patients with residual deficit as a consequence of their disease or treatment to as nearly a normal functioning state as possible.

Approach 1: Develop improved means to provide totally integrated rehabilitation treatment (which) will encompass all aspects of care i.e., physiological, sociological, physiological, etc.) and maximum palliation in all stages of the disease (e.g., management of pain).

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